The Role of Specific Receptor Domains in Signal Transduction by the VIP_2 Receptor

by

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This Thesis is dedicated to my partner, Eve Marie, and to the girls, Eilidh and Isobel, who make it all worthwhile

"...in order to trot out his little scrap of knowledge he will write a book on the whole of physics! From this vice many great inconveniences arise."

Michel de Montaigne

I declare that the studies presented in this Thesis are the result of my own independent investigation, with the exception of the chimaeric receptor constructs which were made by Dr. Eve Lutz, certain cAMP assays which were also carried out by Dr. Eve Lutz and the immunoprecipitation and Western Blotting which were carried out with the assistance of Dr. Rory Mitchell and Mel Johnson respectively. The HA-tagged human VIP_2 receptor cDNA was supplied by Dr. T. MacDonald who modified the original construct created by Dr. Eve Lutz. John Bennie and Sheena Carroll prepared the iodinated ligands for radioimmunoassay and binding studies.

This work has not been and is not currently submitted for candidature for any other degree.

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Abstract

Receptors for the neuropeptides VIP and PACAP belong to a novel sub-family of G protein-coupled receptors, the secretin/calcitonin/parathyroid hormone receptor family. The rat VIP_2 receptor was recently cloned in this laboratory and the present project was carried out to characterise the signalling mechanisms used by this receptor and define the role of crucial receptor domains.

These studies have primarily involved the transient expression of receptors in host cells. The results demonstrated for the first time that both the VIP_1 and VIP_2 receptors can stimulate phospholipase C (PLC) in addition to adenylate cyclase (AC) and that this stimulation occurs by a pertussis toxin(PTx)-sensitive mechanism. Correspondingly, GTPγS modulation of ligand-binding to the VIP_2 receptor in COS 7 cell membranes was shown to be partially PTx-sensitive, suggesting an interaction of the receptor with a PTx-sensitive G protein. An epitope-tagged human VIP_2 receptor was expressed in COS 7 cells and immunoprecipitated with its associated G proteins for Western-blotting studies. Immunoreactivity for $G\alpha_q$, $G\alpha_s$ and a member of the $G\alpha_{i/o/t/z}$ family, other than $G\alpha_{i1}$ or $G\alpha_{i2}$, appeared to be associated with the receptor. The use of specific calcium channel blockers identified a role for receptor-mediated calcium influx in VIP_2 receptor-mediated PLC stimulation.

The PACAP and VIP_2 receptors contain many common structural features but have their own distinct pharmacological profiles. The function of specific domains of the VIP_2 receptor was investigated by creating PACAP/VIP_2 receptor chimaerics and C-terminal truncations of the VIP_2 receptor. These constructs allowed identification of the primary region of the receptor responsible for ligand-binding and the probable site of interaction with a PTx-sensitive G protein and also assessment of any contribution of the C-terminus to AC and PLC stimulation.

A number of further studies were carried out on native receptors. The signalling pathways activated by the endogenous VIP_2 receptor in the GH_3 rat pituitary tumour cell line were investigated. Consistent with previous evidence that a VIP receptor in other cells can stimulate nitric oxide production, VIP_2 receptor-mediated stimulation of nitric oxide synthase, PLC and AC was demonstrated in GH_3 cells. As a result of reports describing the effects of VIP and PACAP on cerebral blood flow the Reverse Transcriptase-Polymerase Chain Reaction technique was used to determine the type of VIP receptor present in rat cerebral microvessels.

Some of the results presented in this Thesis have been published as follows:

Papers

Lutz, E. M., **MacKenzie, C. J.**, Morrow, J., Mitchell, R., Bennie, J., Carroll, S., Clark, E. and Harmar, A.J. (1996) *Chimaeric VIP_2/PACAP receptors reveal that agonist pharmacology but not signal transduction is determined by extracellular domain I,* Annals of the New York Academy of Sciences, 805, pp. 574-578.

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Abstracts

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Abbreviations

AA	arachidonic acid
AC	adenylate cyclase
ACTH	adrenocorticotrophic hormone
cAMP	adenosine 3',5'-cyclic monophosphate
ATP	adenosine triphosphate
AVP	arginine vasopressin
BSA	bovine serum albumin
CHO	chinese hamster ovary
Ci	curies
CNS	central nervous system
$CRAC_i$	calcium-release activated calcium influx
CTx	cholera toxin
Da	daltons
DAG	diacylglycerol
DEAE	diethylaminoethyl
dH_20	distilled water
DMEM	Dulbecco's modified Eagle medium
DMF	dimethylformamide
DMSO	dimethylsulfoxide
cDNA	complementary deoxyribonucleic acid
DNA	deoxyribonucleic acid
dpm	disintegrations per minute
DTT	dithiothreitol
EBSS	Earle's Balanced Salt Solution
EC	extracellular domain
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N,N,N',N'-tetraacetic acid
G protein	guanine nucleotide-binding protein
GAP	GTPase activating protein
GC	guanylate cyclase
GDP	guanosine diphosphate
GH	growth hormone
GHRF	growth hormone-releasing factor

GHRH	growth hormone-releasing hormone
GLP	glucagon-like peptide
GPCR	G protein-coupled receptor
GTP	guanosine triphosphate
GTPγS	guanosine thiotriphosphate
cGMP	guanosine cyclic 5'-monophosphate
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IC	intracellular domain
IP_3	inositol 1,4,5-trisphosphate
MAP kinase	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NGF	nerve growth factor
NO	nitric oxide
cNOS	constitutive nitric oxide synthase
iNOS	inducible nitric oxide synthase
PA	phosphatidic acid
PACAP	pituitary adenylate cyclase-activating peptide
PDBu	phorbol 12,13-dibutyrate
PHI	peptide histidine isoleucine
PHM	peptide hisitidine methionine
PI	phosphatidyl inositol
PI-PLC	phosphoinositide-specific phospholipase C
PIP_3	phosphatidylinositol 3,4,5-trisphosphate
PI 3-Kinase	phosphatidylinositol 3-kinase
PKA	cAMP-dependent protein kinase
PKC	protein kinase C
PKG	cGMP-dependent protein kinase
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PNS	peripheral nervous system
PRL	prolactin
PTx	pertussis toxin
SCN	suprachiasmatic nucleus
TCA	trichloroacetic acid
TM	transmembrane domain
TRH	thyrotropin-releasing hormone

VDCC	voltage-dependent calcium channel
VIP	vasoactive intestinal peptide

Amino Acids

Ala	alanine	Leu	leucine
Arg	arginine	Lys	lysine
Asn	asparagine	Met	methionine
Asp	aspartic acid	Phe	phenylalanine
Cys	cysteine	Ser	serine
Gln	glutamine	Thr	threonine
Glu	glutamic acid	Trp	tryptophan
Gly	glycine	Tyr	tyrosine
His	histidine	Val	valine
Ile	isoleucine		

Declaration

Acknowledgements

Abstract

Abbreviations

Chapter 1

Introduction

1.1 The secretin/calcitonin/parathyroid hormone/parathyroid hormone-related peptide family of G protein-coupled receptors

The vasoactive intestinal peptide(VIP)$_1$ (Ishihara et al., 1991), VIP$_2$ (Lutz et al., 1993) and pituitary adenylate cyclase-activating polypeptide(PACAP) (Morrow et al., 1993) receptors belong to the secretin /calcitonin/parathyroid hormone(PTH)/parathyroid hormone-related peptide (PTHrP) family of G protein-coupled receptors (GPCRs) which also includes the secretin receptor (Ishihara et al., 1991), the calcitonin receptor (Lin et al., 1991), the PTH/PTHrP receptor (Juppner et al., 1991), the GLP-I receptor (Thorens, 1992), the GHRH receptor (Mayo , 1992), the glucagon receptor (Jelinek et al., 1993). These receptors are distinct from the rhodopsin and metabotropic glutamate receptor families of GPCRs and are identified as a family as a result of their sequence homology (Segre & Goldring, 1993).

Receptors of the secretin/calcitonin/PTH/PTHrP receptor family have a high degree of sequence homology with each other and less than 12% homology with the members of other G protein-linked receptor families (Attwood et al., 1991; Probst et al., 1992). All are glycoproteins ranging from 423 to 593 amino acids in length and have an N-terminal hydrophobic signal sequence, a large N-terminal domain, highly conserved N-terminal cysteine residues and N-linked glycosylation sites in extracellular domain(EC)1 and EC2. The putative TM5, TM6 and TM7 regions and the N-terminal portion of the C-terminal tail regions are also highly conserved in the family (Segre & Goldring, 1993).

All GPCRs have common structural features such as the seven hydrophobic domains which are believed to constitute transmembrane-spanning regions, an extracellular amino(N)-terminus and intracellular carboxyl(C)-terminus (see Figure 1.1), and the ability to activate heterotrimeric G proteins by stimulating the rate of

change of GDP for GTP on the α-subunit of the G protein (for review see Baldwin, 1994).

Heterotrimeric G proteins

The extracellular portion of a GPCR such as the VIP_2 receptor recognises specific molecules which on binding cause a conformational change in the intracellular portion of the receptor. Specific heterotrimeric G proteins will then interact with cytoplasmic domains of the receptor and the release of GDP from the Gα subunit will result. GTP is abundant in the cell and will replace the GDP in the binding cleft of the Gα subunit causing a change in the "switch" regions of the Gα subunit and thereby weakening the affinity of Gα and Gβγ for each other and releasing them to act on their respective effectors. The advantage of such a mechanism is in the regulation of the response: the activation of multiple G proteins by an activated receptor means that the response is amplified, a further amplification step may occur at the level of the effector enzyme or ion channel; the short half life of many second messengers means that the duration of the signal can be closely regulated; mechanisms such as phosphorylation can be used to modulate the activity of receptors, G proteins and effectors; the ability of the receptor to be activated by a limited number of agonists and to only interact with specific G proteins provides a high degree of specificity to the response.

There are currently 23 distinct Gα subunits known of size range, 39-52 kDa. The C terminus of the Gα subunit interacts with the receptor (as suggested by the ability of PTx to uncouple G protein receptor-interactions by ADP-ribosylation of a cysteine four residues from the C-terminus) and the first 21 N-terminal amino acids are involved in interacting with the βγ subunits. Gα subunits which contain the MGXXXS consensus myristoylation signal are N-terminally myristoylated ($G\alpha_t$, $G\alpha_i$, $G\alpha_o$, $G\alpha_{gust}$ and $G\alpha_z$). All Gα subunits appear to be palmitoylated at a cysteine

residue near the N-terminus, disrupting myristoylation or palmitoylation prevents both the correct localisation of the Gα subunit and inhibits it's activity. Palmitoylation appears to be a reversible phenomenon which can be used a regulatory mechanism (for review see Birnbaumer & Birnbaumer, 1995).

There are six different Gβ subunits and twelve different Gγ subunits known, giving seventy two potential dimers. However it is known that certain combinations do not occur e.g. $G\beta_2$ with $G\gamma_1$. Denaturing conditions are required to dissociate the Gβγ dimer. The β subunit consists of an N-terminal helix of approximately twenty amino acids and a WD repeat region believed to be a ring of small antiparallel β strands which form a propeller structure (each blade of which consists of four twisted β strands). The WD repeat motif is thought to be important for protein-protein interactions. The size range for the Gβ subunits is 35-39 kDa. The Gγ subunit N terminus forms a coiled-coil with the N terminal non-WD repeat region of the β subunit (the rest of the Gγ subunit stretches out across the surface of the β subunit). The Gγ subunit C terminus contains a CAAX motif which directs the prenylation of the subunit. Gγ1 is farnesylated whereas the others are geranylgeranylated. The cysteine in the CAAX box is the attachment point for these moieties (via a stable thioester bond). The most well-defined function of prenylation is to facilitate membrane association. The geranylgeranyl group is more hydrophobic than the farnesyl group causing a more tightly membrane-associated complex. The N-terminal three amino acids are also cleaved off and the remaining isoprenylated N-terminal cysteine is carboxymethylated. The size range for Gγ subunits is 6-8 kDa (for review see Clapham & Neer, 1997).

Regulation of G protein function

Phosphorylation of $G\alpha_z$ and $G\alpha_{12}$ has been shown to inhibit it's ability to interact with $G\beta\gamma$ subunits thereby extending it's time in the active state (Fields & Casey, 1995; Kozasa & Gilman, 1996).

Regulation of adenylate cyclase by G proteins

All eight characterised isoforms of adenylate cyclase are directly activated by $G\alpha_s$. $G\beta\gamma$ subunits were also found to stimulate ACII and ACIV but not ACV or ACVI. $G\alpha_i$ inhibited ACI, ACV and ACVI but not ACII. $G\alpha_z$ inhibited ACI and ACV. $G\alpha_o$ inhibited ACI but not ACII, ACV and ACVI. $G\beta\gamma$ subunits were found to inhibit ACV and ACVI. The concentration of $\beta\gamma$ subunits required to activate AC is higher than that which results from G_s activation, the activation of G_i or G_o results in much higher levels of $\beta\gamma$ subunits and therefore allows a degree of differentiation between the sources. The activation of ACII and ACIV by $\beta\gamma$ subunits is dependent on coincident activation by $G\alpha_s$. $G_{\alpha i1}$, $G_{\alpha i2}$, $G_{\alpha i3}$ and $G_{\alpha z}$ were all found to be effective inhibitors of ACV and ACVI. $G\alpha_i$ and $G\alpha_o$ were found to be moderately effective inhibitors of ACI, the Ca^{2+}-CaM-stimulated isoform (for review see Sunahara, Dessauer & Gilman, 1996).

A number of AC isoforms contain consensus sequences for phosphorylation by PKA and PKC; ACII and ACV are phosphorylated *in vivo* in response to phorbol myristate acetate causing an activation that is synergistic with the effect of forskolin or $G\alpha_s$ (Kawabe et al., 1994; Jacobowitz & Iyengar, 1994). The action of PKA appears to be inhibitory on ACV and ACVI (Premont et al., 1992).

Regulation of phospholipase Cβ by G proteins

The α subunits of the $G_{q/11}$ and $G_{14\text{-}16}$ subfamily have been demonstrated to stimulate the β isoforms of PLC but not the δ or γ isoforms (see Singer et al., 1997). However PTx-sensitive G proteins have also been reported to stimulate PLCβ isoforms, primarily through the action of Gβγ subunits independently of the Gα subunits (Smrcka & Sternweis, 1993; Boyer et al., 1992; Park et al., 1993). The concentration of βγ subunits required to stimulate PLC is generally found to be two orders of magnitude greater than for $G\alpha_q$ (Exton, 1997). The potency of Gβγ stimulation of purified PLCβ isoforms is: PLC $\beta_3>\beta_2>\beta_1$ (Smrcka & Sternweis, 1993; Park et al., 1993), PLCβ4 is insensitive to Gβγ stimulation (Jiang et al., 1994). The order of potency of G_q stimulation of PLCβ isoforms is: PLC $\beta_1\geq\beta_3>\beta_4>\beta_2$ (Lee & Rhee, 1995). It appears that the β1 isoform appears to be primarily $G\alpha_q$ stimulated, PLCβ_2 is primarily βγ–stimulated and the PLCβ_3 isoform is responsive to both Gα and Gβγ subunits. Most cells contain members of both the $G_{q/11}$ and $G_{i/o}$ families of G proteins; the latter constituting 1-2% of the protein in bovine brain (Sternweis & Robishaw, 1984).

Activation of adenylate cyclase and phospholipase C by G protein-coupled receptors

All members of the secretin /calcitonin/parathyroid hormone(PTH)/parathyroid hormone-related peptide (PTHrP) family of GPCRs are known to couple to adenylate cyclase stimulation by activation of the stimulatory G protein, G_s. A number of these receptors have also been shown to be capable of stimulating an increase in intracellular calcium concentrations and phosphatidyl inositol(PI) hydrolysis. In transfection studies the PTH/PTHrP (Abou-Samra et al., 1992), calcitonin (Chabre et al., 1992; Force et al., 1992) and

GLP-I (Wheeler et al., 1993) receptors were shown to couple to PLC stimulation as well as to adenylate cyclase stimulation. Studies in native cells suggest that VIP receptors (Malhotra et al., 1988), GHRH receptors (Login et al., 1986) and secretin receptors (Trimble et al., 1987) are also capable of mediating PLC stimulation.

GPCRs which do not belong to the secretin/calcitonin/PTH/PTHrP receptor family, such as the dopamine D1 receptor (Liu et al., 1997), TSH receptor (Van Sande et al., 1990), LH receptor (Gudermann et al., 1992), vasopressin V2 receptor and β-adrenergic receptors (Zhu et al., 1994) have also been shown to have this dual signalling capability.

In the majority of cases cAMP stimulation occurs at lower agonist concentrations and requires a lower receptor density than PLC stimulation (Zhu et al., 1994; Van Sande et al., 1995). Adenylate cyclase stimulation is therefore presumed to occur by a more 'tightly-coupled' mechanism than PI hydrolysis i.e. receptor activation of G_s occurs by a more efficient mechanism. It has often been observed that there are more binding sites available for agonist than are required for maximal stimulation of cAMP production, that a 'receptor reserve' exists. This reserve was not normally found when PI hydrolysis stimulated by the same receptors was studied (Zhu et al., 1994; Van Sande et al., 1995).

1.2 Agonists of the secretin/calcitonin/PTH/PTHrP family of G protein-coupled receptors

GPCRs are responsive to a very wide range of agonists, for example photons (in the case of the rhodopsin receptors), opioids, thrombin, catecholamines, cAMP, prostaglandins, large glycopeptide hormones, such as luteinising hormone(LH) and follicle stimulating hormone(FSH), and small peptide hormones such as vasopressin

and oxytocin (for reviews see O'Dowd et al., 1989; Dohlman et al., 1991; Reed, 1992)). Many of the agonists for this family of receptors are peptides with related amino acid sequences and structural similarities (see Figures 1.2 & 1.3). Secretin (Kopin et al., 1990), VIP (Said, 1986), GLP-1, glucagon (Bell, 1986), PHI (Tatemoto & Mutt, 1981), PHM (Itoh et al., 1983), PACAP (Miyata et al., 1989), Growth hormone-releasing factor (GRF/GHRF/GHRH: Rivier et al., 1982), gastric inhibitory peptide and helodermin (Vandermeers et al., 1984) are all small peptides belonging to the same family (see Fig. 1.3). PTH is however much larger at 84 amino acids in length, PTHrP has a high degree of homology with PTH at the N-terminus (eight out of thirteen amino acids being identical) but its remaining sequence is entirely dissimilar (Kronenberg et al., 1993; Martin et al., 1991). The first thirty-four amino acids of PTH have been found to be sufficient for full activity with residues 15-34 being particularly important for receptor binding affinity (Nussbaum et al., 1980). The N-terminal residues are crucial for the stimulation of adenylate cyclase activity (Goltzman et al., 1994; Horiuchi et al., 1983) and it has been demonstrated for various members of the VIP peptide family (Haffer et al., 1991; Gallwitz et al., 1990) that the N-terminus cannot be truncated without affecting the potency of the peptide and its affinity for its receptor.

A brief description of each relevant peptide is given below:

Vasoactive intestinal polypeptide (VIP) is a 28 amino acid peptide which was originally isolated from porcine small intestine by Said & Mutt (1970). The isolated peptide was found to have a structure similar to that of secretin and glucagon (Said & Mutt, 1972). Chromatographically different VIP-like immunoreactivity was isolated from intestinal mucosa (Dimaline & Dockray, 1978; Gafvelin et al., 1988) and subsequently Dimaline & Vowles (1988) observed alternative processing of prepro-VIP in different areas of the GI tract.

Peptide histidine isoleucine (PHI) was also isolated from porcine gut by Tatemoto & Mutt (1981). It has a structure very similar to that of VIP and is now known to be a product of the same precursor polypeptide as VIP (Itoh et al., 1983). VIP and PHI have been identified as coexisting in the intramural neurones of the small intestine (Ekblad et al., 1984).

Peptide histidine methionine (PHM) is a variant of PHI which occurs in humans (Itoh et al., 1983).

Pituitary adenylate cyclase-activating polypeptide(PACAP)-38 is a 38 amino acid peptide first isolated from ovine hypothalamus and found to stimulate adenylate cyclase activity in cultured rat anterior pituitary cells (Miyata et al, 1989).

PACAP-27 is the C-terminally truncated form of the PACAP-38 also isolated from ovine hypothalamus and with similar activity (Miyata et al, 1990).

Helodermin is a 35 amino acid homologue of VIP which was isolated from the venom of the lizard *Heloderma suspectum* (Gila monster). Non-amidated forms occur and are known as helospectins. Helodermin-like immunoreactivity has been identified in a range of mammalian tissues (Robberecht et al., 1985).

Glucagon (29 amino acids) is synthesised in the α cells of pancreatic islets and in non-human mammalian gastric mucosa. This peptide is secreted in response to falling glucose levels and increases the blood glucose concentration. It stimulates glycogenolysis and gluconeogenesis in the liver, increased lipolysis in adipose tissue and increased insulin secretion (Unger & Orci, 1981; Premont & Iyengar, 1987). Glucagon, glucagon-like peptide(GLP)-1 and GLP-2 are derived from the same precusor peptide (Bell et al., 1986).

Glucagon-like peptide(GLP)-1 (37 amino acids) potentiates the effect of glucose on insulin secretion (Creutzfeldt & Ebert, 1985) and stimulates insulin gene transcription (Drucker et al., 1987). Infusion of GLP-1 modulates post-prandial insulin secretion and glucose disposal in non insulin-dependent diabetes mellitus(Nathan et al., 1992).

Parathyroid hormone (PTH; 84 amino acids) primarily acts on the kidney and bone and is the major hormone responsible for regulation of extracellular levels of calcium and phosphorous (for review see; Rosenblatt et al., 1989).

Parathyroid hormone-related peptide (PTHrP) can bind to the same receptors as PTH due to its N-terminal homology but is a larger molecule (Mangin et al., 1989) and fills an as yet undefined role. PTHrP is associated with hypercalcaemia of malignancy and was originally identified in tumours from cancer patients with elevated blood calcium levels (Mangin et al., 1988).

Calcitonin (32 amino acids) increases renal calcium clearance and inhibits osteoclast-mediated bone resorption causing a lowering of serum calcium levels. Calcitonin is a natriuretic agent modulating both nociception and food intake (Azria, 1989; Kopin et al., 1990).

Secretin (27 amino acids) stimulates the exocrine cells of the pancreas to secrete potassium, bicarbonate and various enzymes (Bayliss & Starling, 1982).

Maxadilan is a 61 amino acid peptide from sand flies which has high affinity for the PACAP receptor without having significant affinity for the VIP receptors (Moro & Lerner, 1997).

A number of synthetic peptides selective for VIP_2 receptors have now been developed:

Ro 25-1392 [Ac-Glu^{8},OCH$_3$,Tyr^{10},Lys^{12},Nle^{17}, Ala^{19},Asp^{25},Leu^{26},Lys^{27,28}-vasoactive intestinal polypeptide(cyclo 21-25)] is a cyclic peptide analogue of VIP which is selective for the VIP_2 receptor over the VIP_1 receptor (Xia et al., 1997).

Ro 25-1553 is also a cyclic analogue of VIP which contains a lactam ring (O'Donnell et al., 1994) and has also been demonstrated to be highly selective for the VIP_2 receptor over the VIP_1 receptor (Gourlet et al., 1997).

[Lys^{15},Arg^{16},Leu^{27}]VIP(1-7)GRF(8-27)-NH$_2$ is a VIP/GRF hybrid peptide which is believed to be selective for VIP_1 receptors and does not activate GRF receptors (Gourlet et al., 1997).

Studies with VIP and secretin

A large proportion of the research into the structure and function of this family of peptides has been carried out on the VIP and secretin peptides. A brief review of this work is therefore given here. O'Donnell et al. (1991) replaced residues 24-28 of VIP (which includes residues in the putative helical domain) with Ala residues and found that potency was barely affected, Gourlet et al. (1996) replaced secretin 20-27 with the corresponding sequence of PACAP and found no significant change in affinity. O'Donnell et al. (1991) however found that the whole secretin sequence and VIP (1-27) was required for optimal activity. The N-terminus however could not be truncated without seriously affecting the affinity and activity of the peptides. Deletion of the N-terminal His residue causes a marked reduction in the affinity of VIP and secretin peptides for their receptors (Couvineau et al. (1984); Vilardaga et al. (1994)). Deletion of the first four residues in either sequence markedly reduces the peptides affinity and activity whereas further truncation of VIP or secretin removes their ability to stimulate AC. Musso et al. (1988) first

suggested that the peptides form a C-terminal α-helicoidal structure with segregated hydrophilic and hydrophobic domains as shown in figure 1.2. Holtmann et al. (1995) and Vilardaga et al. (1995) have shown that the receptors N-terminus discriminates between peptides. Gourlet et al. (1996) have carried out studies using C-terminally truncated secretin and VIP peptides and examining their ability to bind to VIP/secretin receptor chimaerics with swapped N-termini. These studies have identified the C-terminus of the peptides as being the region which determines their binding selectivity profile and which interacts with the receptors N-terminus. A study using chimaeric peptides on the same N-terminal swap chimaeric receptors has produced evidence that residues 9-15 of secretin have a major role in interacting with the receptors N-terminus. The authors identify residues Asp^{8}, Tyr^{13} and Lys^{15} as lowering the affinity of PACAP-27 for the secretin receptor whereas residues Thr^{5}, Glu^{9}, Leu^{10}, Glu^{15}, Gly^{16} and certain residues in the 17-20 region (Ala, Arg, Leu, Gln) lower the affinity of secretin for the VIP_1 receptor (Gourlet et al., 1996). Their data suggests that Ser^{9}, Tyr^{10}, Lys^{15}, Gln^{16} and residues in region 17-20 of PACAP-27 are important for PACAP-27 binding to the VIP_1 receptor and that Tyr^{13} and residues 1-7 are not. The evidence for the role of Ile^{5} is inconclusive and it is probable that Ser^{11}, Arg^{12} and Arg^{14} are not required for PACAP-27 binding. These data are broadly supportive of the idea that the N-terminus of the peptide is important for activation rather than binding. A degree of identity between the C-termini of secretin and PACAP-27 makes it difficult to draw any conclusions about their roles from these data.

Both secretin and VIP bind to the rat secretin receptor with high affinity whereas secretin binds with very low affinity to the rat VIP_1 receptor (Holtmann et al., 1995). High affinity VIP binding did not correspond to high activity levels at the secretin receptor, this was not true of secretin itself. The authors suggest that the VIP binding site may be physiologically irrelevant. Chimaeric receptors were

created in which the N-termini and EC1 regions of the receptors were swapped. The secretin N-terminus and EC1 loop proved sufficient to confer secretin receptor-like binding and activity on the VIP_1 receptor whereas the secretin N-terminus on the VIP_1 receptor body created a receptor which had high affinity for neither VIP nor secretin. The VIP receptor N-terminus was enough to give the secretin receptor the ability to cause cAMP generation in response to VIP binding and to decrease the potency of secretin. The addition of the VIP_1 receptor EC1 region further decreased the potency of secretin. The authors therefore found that the N-terminus of the VIP_1 receptor is sufficient for VIP activity, the N-terminus and the EC1 region of the secretin receptor are sufficient for secretin activity and VIP binding and the secretin N-terminus binds neither VIP nor secretin well. Holtmann et al. (1996a) further investigated the necessary sequences and identified the first ten residues of the secretin receptor N-terminus as being necessary for secretin recognition. A construct containing the secretin N-terminus with the N-terminal half of the secretin EC1 region displayed high affinity secretin binding but poor responsiveness suggesting that binding and activation domains were separate. Two basic residues in the secretin EC1 region were identified (His^{189} and Lys^{190}) which assisted in binding secretin, there are indications that Asp^{15} in secretin is involved in binding this motif. A four residue sequence in the N-terminal portion of EC2 (Phe^{257}, Leu^{258}, Asn^{260} and Thr^{261}) was able to substitute for the EC1 but EC3 was not found to be involved in ligand binding.

Holtmann et al. (1996b) found that the rat secretin receptor was capable of high-affinity binding of VIP and that the human receptor was not. There is only one acidic residue which is not conserved between the N-termini of these receptors and when this was mutated by SDM (Asp98Asn) the high affinity binding at the rat receptor was lost. Although binding affinity was high for VIP at the rat receptor the ability to stimulate cAMP was very poor. The authors suggest that the Asp^{98}

residue interacts with the Lys^{15} of VIP (which was previously demonstrated as being important). The equivalent region of the rat VIP_2 receptor contains two aspartate residues, the sequence is not conserved. Vilardaga et al. (1996) identified Lys^{173} in the secretin receptor as being important for the recognition of peptides with an Asp^3 residue such as secretin, VIP and PACAP. This study involved using rat secretin/glucagon receptor chimaerics and peptide analogues which demonstrated that the ability of the receptor to discriminate between ligands that have an Asp at position three or another residue, rested in the EC1 region and could be removed by mutation of Lys^{173}. EC2 appears to have a role to play in binding ligand but not in discriminating between ligands.

1.3 Antagonists of VIP and PACAP receptors

Although no satisfactory antagonists have been found for these receptors a number of antagonists are available which appear to be selective under certain conditions, a brief description of these is given below:

Neurotensin(1-6)VIP(7-28)-NH_2 is a VIP/neurotensin hybrid peptide which displaces 90% of ^{125}I-VIP binding in glial cell cultures and reduced VIP-stimulated cAMP production. It also attenuated sexual behaviours stimulated by VIP (Gozes et al., 1989). In this hybrid the first 6 residues of VIP (HSDAVF) were replaced with the equivalent region of neurotensin.

VIP(10-28) is an antagonist of the human VIP_1 receptor activity in HT29 cells (Turner et al., 1986). It does not cause the down-regulation of VIP receptors in HT29 cells which is observed after treatment with VIP or PHI (Turner et al., 1986) but blocks VIP-evoked AC stimulation in rat pancreatic acini (Dehaye et al., 1986). Cox & Cuthbert (1989) observed that VIP(10-28) caused a small reduction in

electrogenic anion secretion from rat jejunum preparations but did not have any intrinsic agonist activity nor did it behave as a competitive antagonist of VIP in these preparations. Antagonistic properties have however been observed in some systems (Brenneman & Eiden, 1986).

[AcTyr^{1}D-Phe^2]GRF-(1-29)NH$_2$ was observed to be a competitive antagonist of VIP in rat pancreatic plasma membranes (Waelbroeck et al., 1985) this was not the case in rat jejunum (Cox & Cuthbert., 1989).

AcTyr^{1}hGRF-(1-40)-OH was found by Laburthe et al. (1986) to be a competitive antagonist of VIP in rat but not human intestine. Cox & Cuthbert (1989) observed some inhibition of VIP stimulated activity but not competitive antagonism in rat jejunum.

[4Cl-D-Phe^6,Leu^{17}]VIP was ineffective in rat jejunum as a VIP antagonist (Cox & Cuthbert, 1989). In T84 colonic epithelia however VIP-stimulated scc (short circuit current) was inhibited and in exocrine pancreas VIP-stimulated amylase release was inhibited by this compound (Pandol et al., 1986).

[Ac-His^1, D-Phe^2, Lys^{15}, Arg^{16}]VIP (3-7)GRF(8-27)-NH$_2$ is a selective antagonist for the rat and human VIP$_1$ receptors (Gourlet et al., 1997a).

PACAP(6-38) is a potent antagonist of PACAP receptors but has affinity for the VIP$_2$ receptor without being active at the VIP$_1$ receptor (Dickinson et al., 1997).

1.4 Physiological Roles

Since first being identified, VIP and its receptors have been implicated as being central to a wide range of physiological processes. In the interests of clarity a brief description of each distinct major area is given below:

Neurotransmission - VIP is recognised as a neurotransmitter and neuromodulator (for a review see; Rostene, 1984). In autonomic ganglia VIP is found in neurons containing ACh (Hokfelt et al., 1980) and the signal induced by ACh is modulated by VIP (Lundberg et al., 1982; Kawatani et al., 1985). There is evidence that VIP is the neurotransmitter of the non-adrenergic non-cholinergic inhibitory nervous system (Cameron et al., 1983).

Effects on growth of neural tissue - VIP has both trophic and mitogenic effects on embryonic neural tissues (Brenneman et al., 1985; Brenneman & Eiden, 1986) but inhibits growth in certain tumours (Alle et al., 1985) and is also considered to be a part of a new group of neurotrophic factors since it stimulates differentiation and survival of several types of neuronal cells (Pincus et al., 1990; Pence & Shorter, 1990; Watanabe et al., 1990; Deutsch & Sun, 1992; Okumura et al., 1994). VIP also regulates cell death and differentiation of retinal ganglion cells and sympathetic neuroblasts (Kaiser & Lipton, 1990; Pincus et al., 1990).

Electrolyte secretion - VIP is found in myenteric and submucous neurones of the rat intestinal wall (Dimaline et al., 1984; Ekblad et al., 1984) and has long been associated with electrogenic anion secretion /intestinal ion & fluid secretion (Barbezat & Grossman, 1971; Schwartz et al., 1974; Racusen & Binder, 1977; Amiranoff et al., 1978; Binder et al., 1980).

Endocrine function - Immunoreactive PACAP and VIP are found in neurons projecting to the median eminence of the rat hypothalamus (Koves et al., 1991) and

the hypophysial portal blood in rat has been found to contain high levels of both peptides (Rostene, 1984; Dow et al., 1994). VIP has been identified as a physiological regulator of prolactin release from lactotrophs (Rostene, 1984).

i) Regulation of insulin secretion - The VIP_2 receptor is expressed in pancreatic islets and insulin-secreting cell lines (Inagaki et al., 1994). PACAP-like immunoreactivity is present in the nerve fibres innervating pancreatic islets (Fridolf et al., 1992). Kulkarni et al. (1995) demonstrated VIP and PACAP-38 stimulation of insulin and islet amyloid polypeptide (IAP) secretion from HIT-T15 cells. Bertrand et al. (1996) showed VIP_2 receptor mediated stimulation of insulin secretion from rat pancreas and VIP_1 receptor mediated stimulation of glucagon secretion . VIP and PACAP are thought to be potentiators not initiators of glucose-induced insulin release.

ii) Circadian rhythms - The expression of VIP and arginine vasopressin(AVP) mRNA in the suprachiasmatic nucleus(SCN) is modulated by photoperiod (Duncan et al., 1995). Photoperiod information is communicated to the pineal gland from the retina via a multisynaptic pathway which involves a number of hypothalamic nuclei including the SCN (for review see Rusak & Bina, 1990). VIP- and AVP-containing neurons project to the pineal gland from the SCN (Ibata et al., 1989; Kalsbeek et al., 1992; Watts & Swanson, 1990) which is thought to be the circadian pacemaker (Meijer & Reitveld, 1989). VIP production is a central part of the mechanism which, by modulating melatonin secretion from the pineal, has profound effects on fertility in many mammals (McArthur et al., 1997; Duncan et al., 1995). VIP stimulates serotonin N-acetyl transferase activity (Klein & Berg, 1970; Klein & Weller, 1970; Klein & Weller, 1973; Yuwiler, 1987) and melatonin synthesis and release from the pineal gland (Spessert, 1993; Simmoneaux et al., 1993).

Smooth muscle relaxation - VIP receptors in dispersed gastric smooth muscle cells stimulate a constitutive Ca^{2+}/calmodulin dependent NOS which causes the activation of guanylate cyclase and smooth muscle relaxation as a result of cGMP-dependent protein kinase activity (Murthy et al., 1993). VIP release from myenteric neurons is partly responsible for relaxation of gastric (Grider & Rivier, 1990; Gu et al., 1992), intestinal (Grider & Makhlouf, 1986) and sphincteric smooth muscle (Goyal et al., 1995). VIP stimulates the relaxation of visceral and vascular smooth muscle (Li & Rand, 1990; Gaw et al., 1991; Grider et al., 1992).

i) Vasculature - Radioimmunoassay and immunohistochemical methods have shown VIP innervation of cerebral blood vessels (Larsson et al., 1976; Edvinsson et al., 1981). In rat, PACAP and VIP have similar potency for decreasing systemic arterial pressure (Nandha et al., 1991; Absood et al., 1992) and relaxing tail or mesenteric arteries (Absood et al., 1992; Huang et al., 1993). VIP caused vasodilation of isolated cerebral artery or vein through direct application and increased cerebral blood flow when applied intra-arterially to baboons (Edvinsson et al., 1981). Anzai et al. (1995) observed similar concentration-dependent relaxations in canine basilar arteries and rat intracerebral arterioles on addition of PACAP-27, PACAP-38 and VIP. The VIP_1 receptor is involved in the relaxation of blood vessels in the rat pancreas (Bertrand et al., 1996).

ii) Erectile function - VIP has been suggested as being the neurotransmitter that mediates penile erections and which can also stimulate sexual behaviours (Gozes et al., 1989). VIP is present in nerve fibres innervating the cavernous smooth muscle and blood vessels and is elevated during erection. Injection of exogenous VIP causes erection in man (Ottesen et al., 1984; Dixon et al ., 1984) and VIP levels have been shown to be decreased in impotent men (Gu et al., 1984).

iii) Airway smooth muscle - Ollerenshaw et al. (1989) postulated that the absence of VIP in the lung is responsible for the development of asthma since imunohistochemical methods failed to detect VIP in samples from asthmatic patients. Human lung contains high levels of VIP (Polak & Bloom, 1982) and VIP immunoreactive neurons (Carstairs & Barnes, 1986). VIP_1 and VIP_2 receptor mRNA was found in the bronchi and the vascular epithelium of the lung (Usdin et al., 1994). O'Donnell et al. (1994a;1994b) found that the VIP analogue, Ro 25-1553, was a more potent bronchodilator than isoproterenol or salbutamol and was effective in relaxing airway smooth muscle and reducing inflammation in guinea pig lung *in vitro* and *in vivo*.

Cerebral energy metabolism - VIP stimulates glycogenolysis in the cerebral cortex (Magistretti et al., 1981). Stimulation of VIP receptors on astroglia has also been shown to increase glycogenolysis and to change the cells morphology from flat to predominantly process-bearing cells (Magistretti, 1993).

Immune System - VIP has potent immunoregulatory properties in both experimental animals and humans (Ottaway, 1991). VIP affects murine monocyte chemotaxis (Bondesson et al., 1991), inhibits the proliferative response of murine lymphocytes to mitogens (Ottaway & Greenberg, 1984; Stanisz et al., 1986; Ottaway, 1987; Boudard & Bastide, 1991) and modulates the production of immunoglobulins (Stanisz et al., 1986) and IL-2 (Ottaway, 1987) in response to Concanavilin A stimulation (an agglutinating and mitogenic protein). Matthew et al. (1992) have shown that VIP stimulates the release of IL-5 from activated T cells in murine schistosomiasis.

In human lymphocyte cultures VIP can modulate the production of immunoglobulins in response to mitogen stimulation (Drew & Shearman, 1985), T

cell proliferative responses (Nordlind & Mutt, 1986) and the expression of natural killer cell activity (Drew & Shearman, 1985; Rola-Plesczynski et al., 1985).

The VIP_2 receptor has been demonstrated to have a role in IL-6 production and haematopoiesis in rat bone-marrow derived stromal cells (Cai et al., 1997). VIP and PACAP are secretagogues for IL-6 in anterior pituitary cells as are cytokines (Spangelo et al., 1991; Matsumoto et al., 1993). VIP-immunoreactive nerve fibres and lymphoid cells have been shown in both primary and secondary lymphoid organs (Felten et al., 1985; Ottaway et al., 1987; Fink & Weihe, 1988; Gomariz et al., 1990, Gomariz et al., 1992) and the existence of VIP receptors has been documented on various types of immune cells (Guerrero et al., 1981; Danek et al., 1983; Beed et al., 1983; Ottaway & Greenberg, 1984; Calvo et al., 1986; Finch et al., 1989; O'Dorisio et al., 1989; Ottaway et al., 1990).

1.5 Receptor Distribution

1.5.1 VIP receptors

VIP_1 and VIP_2 receptors appear to be distributed in what is generally a mutually exclusive fashion in the brain. The VIP_1 receptor is found exclusively in the cerebral cortex and hippocampus whereas the VIP_2 receptor is found in a number of areas of the diencephalon and brainstem (see Table 1.1, Figures 1.4 & 1.5). These receptors are also largely distributed in a complementary fashion in other tissues (see Table 1.1 & 1.2). The VIP_2 receptor is found in stomach and duodenal muscle and cells in the pancreatic islets (whereas the VIP_1 receptor is only found in associated cells). In the spleen, testes and ovary only VIP_2 receptor is found at significant levels (in the parenchymal cells). In the kidney, VIP_1 and VIP_2 receptors are present in different parts of the nephron. In the thymus and adrenal,

the cortex and medulla contain both receptors but in varying amounts. Uterus and blood vessels appear to contain both receptors but they are quite possibly present in different cell types (Usdin et al., 1994). Wei & Mojsov (1996) failed to detect any VIP_1 receptor mRNA in human pancreas by RNase protection assay but did find VIP_2 receptor mRNA in human skeletal muscle (see Table 1.3). Adamou et al. (1995) also found two VIP_2 receptor transcipts to be present in human skeletal muscle, there are currently no other published examples of VIP or PACAP receptors being detected in skeletal muscle. The difficulty in making a muscle preparation which is free of blood vessels must be considered with respect to these data. Human adipocytes express VIP_1, VIP_2 and PACAP receptors (Wei & Mojsov, 1996) and rat bone marrow-derived stromal cells strongly express VIP_2 receptor mRNA, rat bone marrow tissue expresses VIP and PACAP mRNA (Cai et al., 1997). Cultured cells which express VIP or PACAP receptors are listed in Table 1.4.

Fahrenkrug (1993) suggested that VIP in the brain was involved in regulating coupling between energy metabolism, blood flow and neuronal activity. The association of the VIP_1 receptor with the cortex, hippocampus and amygdala indicate that its effects may be primarily on behaviours and higher cortical functions. The presence of VIP_2 receptor mRNA in the brain stem motor nuclei and thalamus suggests that it may be involved in sensory information processing. VIP_2 receptor mRNA is present at relatively high level in the anterior pituitary and the hypothalamus where VIP_1 receptor mRNA is either absent or present at very low levels (Usdin et al., 1994). It is therefore not surprising that the VIP_2 receptor has been found to be important for the regulation of secretion from the pituitary (Rostene, 1984). VIP_2 receptor mRNA has been found to be abundant in the suprachiasmatic nucleus and VIP_1 receptor mRNA in the pineal gland (Usdin et al.,

1994). VIP has been found to play a central role in the regulation of circadian rhythms (Duncan et al., 1995).

1.5.2 PACAP receptors

A high density of PACAP receptors was detected in rat brain and spinal cord (Gottschall et al., 1990; Lam et al., 1990; Cauvin et al., 1991; Gourlet et al., 1991), bovine brain (Ohtaki et al., 1990), rat anterior pituitary (Gottschall et al., 1990), cultured rat astrocytes (Tatsuno et al., 1991; Nilsson, 1994), the AR4-2J pancreatic acinar cell line (Buscail et al., 1990; Gourlet et al., 1991), retina (Nilsson, 1994), a rat phaeochromocytoma cell line, PC12 and a human neuroblastoma cell line, NB-OK (Cauvin et al., 1990). A monocytic leukaemia cell line, THP-1, also expresses PACAP receptor (Chedeville et al., 1993) as do human adipocytes (Wei & Mojsov, 1996).

PACAP receptor binding sites were identified in the hypothalamus, brainstem, cerebellum, cerebral cortex and basal ganglia of both rat and human (Lam et al., 1990; Suda et al., 1991). Masuo et al. (1991) used autoradiography to demonstrate a high density of PACAP receptor binding sites in rat anterior pituitary, hippocampus and superior colliculus, moderate to high labelling in the periaqueductal gray matter, substantia nigra pars compacta and habenula and moderate labelling in the hypothalamus, ventral tegmental area, mammilary body and medial geniculate nucleus. A high density of binding sites was also identified in the spinal trigeminal nucleus in the brainstem, lamina I-III and X in the spinal cord and the intermediolateral column of the thoracic cord. Legradi et al. (1994) found that in all other areas of the spinal cord the white matter was unlabelled and the grey matter moderately labelled.

The results of Morrow et al. (1993) and Spengler et al. (1993) are tabulated in Table 1.5. This additionally demonstrates the presence of type I PACAP receptor mRNA in rat testes, adrenal gland, olfactory bulb and occipital cortex. Spengler et al. (1993) identified a number of splice variants of the PACAP receptor as described in Section 1.6.3. Spengler et al. (1993) found the PACAP receptor without insert (see Figure 1.5) to be dominant in all tissues tested except in olfactory bulb, which had low 'hip' isoform levels and high 'hop' levels, and in testes and adrenal gland which were predominantly 'hop'. The hippocampus had low 'hip' isoform levels. The distribution of PACAP and VIP receptors is distinct but overlapping. The PACAP receptor is expressed predominantly in the brain and very little in peripheral tissue and is present at higher levels in the brain than the VIP receptors (Ishihara et al., 1992). This was demonstrated by Masuo et al. (1992) who compared [^{125}I]PACAP-27 and [^{125}I]VIP binding sites in rat brain sections, these authors found that [^{125}I]PACAP-27 binding sites appeared to be more numerous than [^{125}I]VIP binding sites in all tissues tested, the difference being particularly striking in the substantia nigra and areas of the cerebral cortex, where the levels of [^{125}I]VIP binding were low. The VIP receptors are expressed at higher levels in various peripheral tissues.

1.6 Isolation and Cloning of the Receptors

Paul & Said (1987) solubilised VIP receptors from guinea pig lung using the zwitterionic detergent CHAPS. They used high performance gel filtration to separate the functional solubilised receptors and found that they had two active fractions. One fraction had a Stokes radius of 5.9±0.1 nm and an IC_{50} for homologous displacement of ^{125}I-VIP binding of 240 pM. The other fraction had a Stokes radius of 2.3±0.1 nm and an IC_{50} for homologous displacement of ^{125}I-VIP

binding of 1.2 μM. The authors found that a large proportion of the high affinity binding complex could be dissociated into a low affinity binding form by treatment with a higher CHAPS concentration and that the non-hydrolysable GTP analogue guanyl-5'-yl-imidophosphate (Gpp(NH)p) had a similar effect. This indicated that the high affinity binding fraction was a G protein-complexed receptor (larger size, higher affinity for ligand) and the lower affinity binding fraction contained a receptor without associated G protein. It is interesting that PHI and GRF were respectively 88- and 23-fold less potent than VIP at displacing ^{125}I-VIP binding from the high affinity receptor complex whereas PHI was 31-fold less potent than VIP at displacing binding from the low affinity receptor and GRF was 23-fold more potent, suggesting that the high and low affinity states of the receptor have different ligand binding preferences. Couvineau et al. (1990) used affinity chromatography to purify a VIP receptor (53 kD) from porcine liver. Sreedharan et al. (1991) believed that they had cloned a human VIP receptor from the pre-B lymphoblastic cell line Nalm 6 and the colon carcinoma cell line HT-29. They cloned the rat homologue of this receptor to confirm their results but it was found that when the cDNA was expressed in COS cells there was no specific binding of ^{125}I-VIP.

1.6.1 VIP_1 receptor

i) Rat

Ishihara and coworkers cloned the rat secretin receptor (Ishihara et al., 1991) and then went on to clone the rat VIP_1 receptor from a rat lung cDNA library (Ishihara et al., 1992). The authors used rat secretin receptor cDNA for cross-hybridisation and isolated the rat receptor cDNA. The sequence contains an open-reading frame of 459 amino acids with a N-terminal signal sequence of 30 amino acids The mature receptor is therefore 429 amino acids long with a mass of 49 kD which agrees reasonably well with the mass of 53 kD determined by Couvineau et

al. (1990) in porcine liver, when glycosylation is considered. The receptor contains seven putative transmembrane-spanning domains as determined by a hydrophobicity plot (Kyte & Doolittle, 1982). There is 48% homology at the amino acid level with the rat secretin receptor (Ishihara et al., 1991), 33% with the porcine calcitonin receptor (Lin et al., 1991) and 39% with the opposum receptor for PTH and PTHRP (Juppner et al., 1991).

The cDNA was expressed in mouse COP cells and membranes were prepared for ligand binding studies. A Scatchard analysis of ^{125}I-VIP binding revealed two distinct binding sites with dissociation constants of 173 pM and 21 nM at concentrations of 4.1 and 53 pmol/mg protein respectively, similar to those obtained in rat lung membrane fractions (Leroux et al., 1984). Displacement of ^{125}I-VIP binding gave IC_{50}s of: 1.0 nM for PACAP-38; 2.5 nM for PACAP-27; 3.0 nM for VIP; 6.0 nM for helodermin and PHM. Secretin was approximately 100-fold less potent than VIP and glucagon at 2.5 μM had no effect on VIP binding. These results are very similar to those from rat lung. The cDNA was expressed in COSGs1 cells which overexpress the α subunit of the rat G_s protein (Ishihara et al., 1991). Stimulation of cAMP production was comparable to that obtained with the secretin receptor. EC_{50}s were: 0.28 nM for PACAP-38; 0.42 nM for PACAP-27; 0.57 nM for VIP. Helodermin and PHM had EC_{50}s around 1 nM, the EC_{50} for secretin was around 9 nM whereas glucagon hardly caused any stimulation.

ii) Human

In 1993 Sreedharan et al. succeeded in cloning the human VIP_1 receptor from a human colon carcinoma cell-line (HT29). A PCR-derived secretin receptor probe was used to screen the HT29 cDNA library. A clone was selected which encoded a 457 amino acid protein with a putative molecular mass of 52 kD. Northern blot analysis using a clone containing the partial VIP_1 receptor sequence revealed 2.8 kb

transcripts in human lung, HT29 cells and Raji B-lymphoblasts with weaker expression in the human brain, heart, kidney, liver and placenta. At the nucleotide level the sequence had 85% identity with the rat VIP_1 receptor and 56% with the rat secretin receptor. At the amino acid level the homology was 84%, 44% and 35% with the rat VIP_1, secretin and GLP-1 receptors respectively. The protein is missing the Gln^{134} and Ile^{290} which are present in the rat receptor . There are eight cysteine residues in the N-terminus and four conserved potential N-glycosylation sites (Asn^{58}, Asn^{69}, Asn^{100} and Asn^{290}). The clone was expressed in COS 7 cells and ^{125}I-VIP binding was observed to occur with a Kd of 0.8 nM as determined by Scatchard analysis. The construct was then expressed in HEK-293 cells where cAMP production was measured with and without $G_s\alpha$ cotransfection. In the absence of additional $G_s\alpha$, 10 µM VIP caused a 3-fold stimulation of intracellular cAMP levels and a 14-fold stimulation in it's presence. EC_{50}s were not determined but it appears that VIP, PACAP-38 and PHM were of similar potency (around 1 nM) with secretin approximately an order of magnitude less potent.

Couvineau et al. (1994) isolated two cDNA clones from a human jejunal epithelial cell cDNA library which were divergent only in their N-termini. The clone hIVR8 encodes a 460 amino acid protein the sequence of which is 81% homologous with the rat lung VIP_1 receptor (Ishihara et al., 1992). Hydrophobicity analysis predicts the typical seven transmembrane-spanning regions. There is a 30 amino acid N-terminal signal sequence (cleavage between Ala^{30} and Ala^{31}) indicating a mature protein 430 amino acids in length. The protein has a predicted mass of 49 kD after cleavage of the signal sequence; affinity cross-linking studies give a mass of 67kD probably as a result of glycosylation. The sequence has four potential N-glycosylation sites; Asn^{58}, Asn^{69} and Asn^{100} in the N-terminus and Asn^{293} in EC2. The sequence contains one cysteine each in EC1 and EC2 (Cys^{208} and Cys^{288}), a potential PKA phosphorylation site on Ser^{451} in the C-terminal tail and a site for

PKC at Ser^{250} in IC2. When expressed in COS 7 cells hIVR8 facilitated specific ^{125}I-VIP binding at a single site with a dissociation constant of 0.6 nM and a Bmax of 211 fmol/mg membrane protein. The rank order of potency for VIP and its related peptides was: VIP = PACAP-27 > PACAP-38 > helodermin > hGRF = PHM > secretin. hIVR8 has 48% sequence identity with the rat VIP_2 receptor (Lutz et al., 1993); rat PACAP receptor, 54% (Pisegna & Wank, 1993); human GRF receptor, 42% (Gaylinn et al., 1993); rat liver glucagon receptor, 39% (Jelinek et al., 1993); opossum kidney PTH receptor, 37% (Juppner et al., 1991); rat pancreatic GLP I receptor, 37% (Thorens, 1992); porcine kidney calcitonin receptor, 29% (Lin et al., 1991).

The other clone hIVR5, as mentioned on the previous page, was identical in sequence to hIVR8 except for its N-terminal 67 amino acids This clone was 495 amino acids in length. When expressed in COS 7 cells this clone caused no specific binding of ^{125}I-VIP or ^{125}I-PACAP-27. Couvineau et al. suggest that the two forms of the protein may result from alternative splicing of the same mRNA transcript and that proteolytic maturation may produce a functional receptor. There have been no further developments to date regarding this hypothesis.

1.6.2 VIP_2 receptor

i) Rat

The rat VIP_2 receptor (see Figure 1.1) was cloned by Lutz et al. (1993) from a rat olfactory bulb and pituitary cDNA library. Oligonucletide primers corresponding to conserved regions of TMIII and TMVII of the secretin, calcitonin and PTH receptors were used to amplify fragments from rat pituitary which were subcloned and sequenced. One fragment contained a 500 bp insert which represented a new member of this family. This fragment was used as a hybridising

probe for Northern blot analysis of rat tissue. This revealed a 3.5 kb transcript in pituitary and other brain regions with the strongest band appearing in the olfactory bulb sample. A rat olfactory bulb cDNA library was therefore screened with probes for the novel sequence and three clones were isolated, one of these clones contained the complete open reading frame giving a protein of 437 amino acids and a predicted molecular mass of 49,519. The protein contains a predicted hydrophobic 22 amino acid signal sequence with a cleavage point between Pro^{22} and Glu^{23}. The hydrophobicity plot predicts the expected seven transmembrane spanning domains. The eight cysteine residues conserved in the rat VIP_1, secretin, GHRH and PACAP receptors and human VIP_1 are also conserved in this receptor. There are putative N-glycosylation sites at Asn^{57}, Asn^{87} and Asn^{91} in the N-terminus. There is 50% sequence identity at the amino acid level with the rat VIP_1 and PACAP receptors with the highest homology occurring in the transmembrane regions. The N- and C-termini are highly divergent in sequence.

The construct was expressed in COS 7 cells in order to assess the receptor's ability to stimulate cAMP production. PACAP-38, PACAP-27, helodermin and VIP were of similar potency (EC_{50}s of 0.18 nM, 0.43 nM, 0.25 nM and 0.17 nM respectively), PHI was less potent (2.14 nM) and CRF, CGRP, secretin and glucagon had negligible effect. From a single dose it was assessed that rGHRH was less potent than PHI. The rank order of potency being; VIP ~ P-27 ~ P-38 ~ helodermin > PHI >> rGHRH.

In situ hybridisation of VIP_2 receptor mRNA in rat brain revealed its presence predominantly in the suprachiasmatic nucleus and paraventricular nucleus of the hypothalamus (with lower levels in the supraoptic nucleus), the mediodorsal and ventral thalamus, the CA1 and CA3 subfields of the hippocampus and in the dentate gyrus. The mRNA was present in all layers in the olfactory bulb except the

external plexiform layer. Low levels were observed in the pituitary. No labelling was observed in the cerebral cortex or cerebellum.

ii) Human

Adamou et al. (1995) cloned the human VIP_2 receptor from a human placental cDNA library. They used degenerate oligonucleotide primers based on conserved regions in the first intracellular loop and seventh transmembrane regions of the secretin, PTH and glucagon receptors to amplify a fragment encoding the human VIP_2 receptor. The open-reading frame encodes a 438 amino acid protein with a predicted molecular weight of 49.5 kD. The sequence contains a predicted signal sequence of 20 amino acids with a cleavage point between Ser^{20} and Ile^{21}. In addition to the hydrophobic signal peptide, the sequence contains another seven hydrophobic regions which are putative transmembrane spanning regions. Cleavage of the signal peptide would give a predicted molecular weight of 46.5 kD. There are 3 potential N-glycosylation sites in the N-terminus; Asn^{58}, Asn^{88} and Asn^{92}. The human and rat receptors are 85% identical at the amino acid sequence level and 82% identical at the level of nucleotide sequence. The human VIP_2 receptor is 52% homologous to the human PACAP receptor (Ogi et al., 1993); 49% with the human VIP_1 receptor (Sreedharan et al., 1993); 48% with the rat secretin receptor (Juppner at al., 1991); 41% with the human GHRH receptor (Mayo, 1992); 49% with the human PTH and glucagon receptors (Schneider et al., 1993; MacNeil et al., 1994). Scatchard analysis of ^{125}I-VIP binding showed a single high affinity binding site (K_d, 63.6 pM; B_{max}, 34.7 fmol/mg protein) when the cDNA was transiently expressed in COS 7 cells. VIP and PACAP-38 displaced ^{125}I-VIP binding with IC_{50}s of 0.93 nM and 6.2 nM respectively. Secretin was very poor at displacing ^{125}I-VIP binding.

1.6.3 PACAP Receptors

i) Rat

Morrow et al. (1993) cloned the PACAP receptor from a rat olfactory bulb cDNA library using degenerate oligonucleotide primers for the conserved regions in TM3 and TM7 of the calcitonin, secretin and PTH receptors. The receptor was cloned in two splice variant forms, with and without an 84 bp exon (28 amino acids: see Figure 1.10). The first 19 N-terminal amino acids of this receptor were predicted to be a signal sequence with a cleavage site between Ala^{19} and Met^{20}. The mature receptor without the 28 amino acid insert in IC3 was therefore 448 amino acids long. Potential N-glycosylation sites were identified at positions 47, 59 and 116 on the N-terminus, position 299 in EC2 and position 342 in IC3. It is worth noting that potential phosphorylation sites were found at Ser^{365} and Thr^{375} in the 28 amino acid insert portion of IC3. They observed that the cDNA when expressed transiently in COS 7 cells was functionally coupled to adenylate cyclase stimulation with the following potency of ligands: PACAP-38 = PACAP-27 > VIP > PHI. Hashimoto et al. (1993), Hosoya et al. (1993), Pisegna & Wank (1993), Spengler et al. (1993) and Svoboda et al. (1993) also went on to clone this receptor and a number of splice variants from rat tissue. Spengler et al. (1993) isolated five splice variants of the PACAP receptor from a new-born rat colliculi cDNA library. They used the transcriptional induction of a cAMP-responsive luciferase gene as a novel expression cloning strategy to identify receptors positively coupled to adenylate cyclase. The DNA and reporter construct were cotransfected into LLC PK1 cells which do not have endogenous receptors responsive to PACAP or VIP. Two cDNAs were cloned which had identical sequences except for the presence or absence of an 84 bp (28 amino acids) cassette in the C-terminal portion of the third

intracellular loop. The authors used primers for the 3' and 5' untranslated regions of the isolated cDNAs to isolate an additional 3 clones from a rat genomic library. The clones were divergent only in the presence or absence of two 84 bp cassettes in the C-terminal portion of the third intracellular loop (see figure 1.9). The authors found that the exon-intron boundaries were in excellent agreement with the consensus exon-intron junction sequences and also identified two contiguous splice acceptor sites used to generate the hop1 and hop2 (which contains a serine residue missing from hop1) variants of the PACAP receptor. The presence of introns in the PACAP receptor gene is of particular interest since many G protein-coupled receptor genes lack introns. There are however a number of examples now of secretin family receptors genes containing introns (Mayo, 1992). The first 19 amino acid residues in the N-terminus are believed to encode a signal sequence (Hosaya et al., 1993). The signalling characteristics of the PACAP receptor splice variants will be discussed later.

ii) Human

The human PACAP receptor was cloned by Ogi et al. (1993) and consists of 525 amino acids with a predicted 77 residue N-terminal signal sequence. The length of the mature receptor is therefore 448 amino acids, the same length as the rat receptor without an insert. There is 92.5% homology at the level of amino acid sequence between the rat and human isoforms of this receptor. The gene for the human receptor has been localised to chromosome 7 by using human-mouse somatic cell hybrids. Human genomic DNA and the hybrid cells containing chromosome 7 both produced bands after Southern blot analysis using the PACAP receptor DNA as a probe (Ogi et al., 1993).

iii) Bovine

Ohtaki et al. (1993) used a synthetic oligonucleotide probe based on the sequence of the N-terminus of the purified bovine PACAP receptor to clone two splice variants of the PACAP receptor from a bovine brain cDNA library. One clone had an open reading frame of 513 amino acids with the first 37 N-terminal residues being a signal sequence (based on the sequence of the purified receptor). This means that the clone is the same length as the hop1-containing rat and human PACAP receptors. There were four potential N-glycosylation sites in the extracellular loops and another in the putative EC3. The other clone was shorter by 28 amino acids and corresponds to the short PACAP receptor which has no insert.

1.7 Nomenclature

The International Union of Pharmacology (IUPHAR) Committee on Receptor Nomenclature have proposed an alternative nomenclature for the VIP_1, VIP_2 and PACAP receptors, it is suggested that they be known as the $VPAC_1$, $VPAC_2$ and PAC_1 receptors respectively (Harmar et al., 1998).

1.8 Aims of this Study

Despite the cloning of the VIP_1 and VIP_2 receptors from a number of different species, the signalling characteristics of the VIP receptors has not been extensively characterised. The rat VIP_2 receptor was cloned by Dr. Eve Lutz in this laboratory and this study was initiated with two main aims:

i) to characterise the ability of the rat VIP_2 receptor to activate intracellular signalling cascades in a heterologous expression system (transient transfection of COS 7 cells) and in cells expressing endogenous VIP_2 receptors (the GH_3 rat anterior pituitary cell line).

ii) to investigate the function of specific receptor domains through the use of chimaeric PACAP/VIP_2 receptors and C-terminally truncated VIP_2 receptors. Constructs were created by Dr. Eve Lutz in this laboratory and exploited the homology between the VIP_2 and PACAP receptors to allow an investigation of the role of receptor domains in determining the VIP_2 and PACAP receptors signalling and ligand binding characteristics.

Fig. 1.1 Identity between the rat $VIP_{2\ wt}$ receptor and PACAP receptor sequence.

The red residues are those conserved between the $VIP_{2\ wt}$ and PACAP receptors. The blue bar indicates the site of cleavage of the signal sequence.

'*' indicates the putative N-linked glycosylation sites.

Fig. 1.1

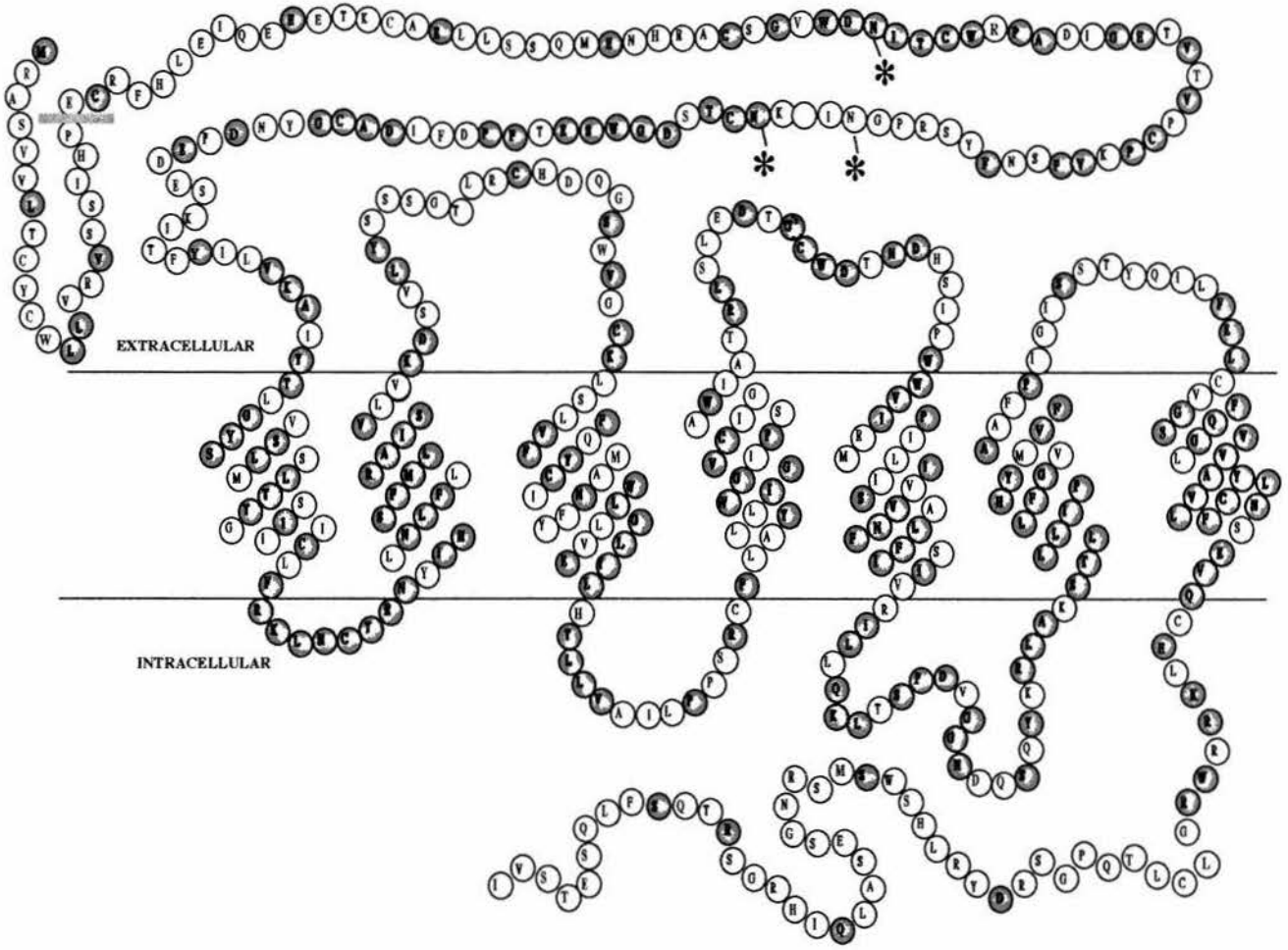
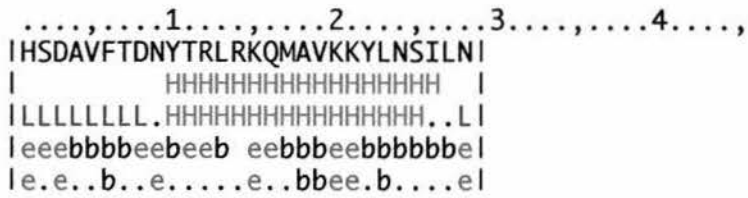


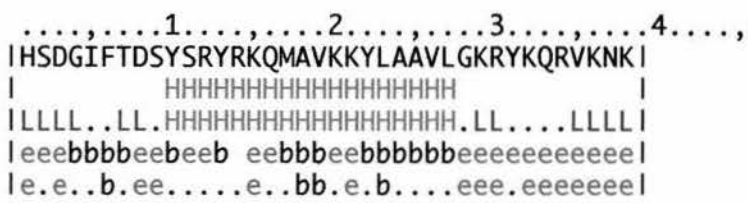
Fig. 1.2

Sequence and predicted secondary structure of peptide agonists

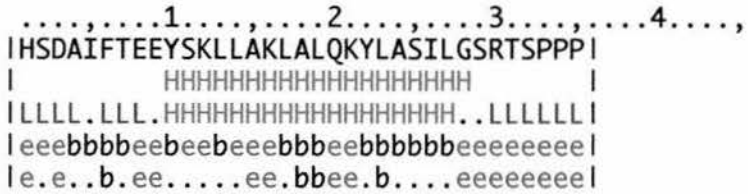
VIP



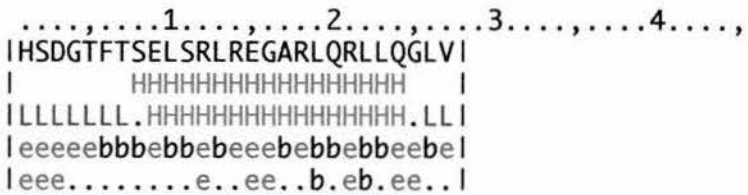
PACAP-38



Helodermin



Secretin



PHI

Secondary structure

The first two lines below the sequence are an estimate of secondary structure. The second line of prediction represents a subset with a higher expected average accuracy (> 82%).

E represents a probable extended strand of the sequence

H represents a probable helix

L or a space in the first line, represents a probable looping portion of the sequence

. represents a residue for which no confident prediction can be made

Solvent Accessibility

The third and fourth lines are estimates of the solvent accessibility of the individual residues. The fourth line is a subset with an average expected correlation > 0.69.

b represents a 'buried' residue

e represents an 'exposed' residue

. represents a residue for which no confident prediction can be made

A system of neural networks perform the prediction task from multiple sequence alignment input. Secondary structure is determined by the PHDsec programme and solvent accessibility by the PHDacc programme on the PHD server at EMBL-Heidelberg. A full description of the methods used can be found in Rost & Sander, 1993; Rost et al., 1994; Rost & Sander, 1994.

Fig. 1.3

VIP family peptide sequences

VIP	(o/h/r/p/ra/go/do/b)	HSDAVFTDNYTRLRKQMAVKKYLNSILNa
PACAP-27	(o/h/p/f)	HSDGIFTDSYSRYRKQMAVKKYLAAVLa
PACAP-38	(o/h/r)	HSDGIFTDSYSRYRKQMAVKKYLAAVLGKRYKQRVKNKa
Helodermin	(hs)	HSDAIFTEEYSKLLAKLALQKYLASILGSRTSPPP
Secretin	(b/p/gp/o)	HSDGTFTSELSRLRDSARLQRLLQGLVa
Glucagon	(b/h/p)	HSQGTFTSDYSKYLDSRRAQDFVQWLMNT
PHI	(b/o/p)	HADGVFTSDYSRLLGQLSAKKYLESLIa
PHM	(h)	HADGVFTSDFSRLLGQLSAKKYLESLMa
GIP	(p)	YAEGTFISDYSIAMDKIRQQDFVNWLLAQKGKKSDWKHNITQ
GRF(1-44)	(p)	YADAIFTNSYRKVLGQLSARKLLQDIMSRQQGERNQEQGARVRLa

The table shows the amino acid sequences of the VIP family peptides. o, ovine; h, human; r, rat; p, porcine;, ra, rabbit; go, goat; do, dog; b, bovine; gp, guinea pig; hs, *Heloderma suspectum* (Gila monster); a, $-NH_2$. Certain residues are coloured red to emphasise the conservation of sequence between peptides.

Table 1.1

Distribution of VIP_1 and VIP_2 receptor mRNA in the rat: as assessed by *in situ* hybridisation

i) VIP_1 receptor distribution

Cerebral cortex
    Layers III and V
Several amygdaloid nuclei
Hippocampus
Olfactory bulb
    External plexiform layer
Thalamus
    Ventrolateral portion of anteroventral nucleus
Pineal
Amygdala
    No labelling
Hypothalamus
    No labelling
Midbrain, pontine or medullary neurons
    No labelling
Choroid plexus
    Some cells of pia mater

Stomach & Duodenum
    No labelling
Spleen
    Light labelling of certain cells
Kidney
    High epithelium of proximal and distal tubules with renal cortex and some juxtaglomerular cells
Thymus
    Mostly in cortical areas
Adrenal
    Adrenal medulla and cortex highly labelled
Pancreas
    Walls of blood vessels with both thick and thin muscular layers
Liver
Lung
    Vascular epithelium and large and moderately sized bronchii epithelium
Testes
    Cells within connective tissue septa
Ovary
    No labelling

Table 1.1 (cont.)

Uterus
    Intense labelling of smooth muscle cells
Pituitary
    Little or no labelling

ii) VIP_2 receptor distribution

Thalamus
    Except lateral ventral nucleus and the ventrolateral portion of anteroventral nucleus
Hypothalamus
Midbrain
Brainstem
Olfactory bulb
    Internal granular layer
Cerebral cortex
    Layer VI
Medial geniculate body
    Heavy labelling
Intermediate subdivision of central amygdaloid nucleus
    Heavy labelling
Hippocampus
    Heavy labelling
Nucleus accumbens
Bed nucleus of stria terminalis
Diagonal band of Broca
Hypothalamus
    Medial preoptic areas
    Lateral preoptic areas
    Organon vasculosum laminae terminalis
    Suprachiasmatic nucleus
        ventral subdivision (heavy labelling)
        periventricular portion
    Magnocellular hypothalamic nuclei
        Paraventricular nucleus
        Supraoptic nucleus
        Accessory magnocellular nucleus
        Arcuate nucleus
        Posterior hypothalamic nucleus (heavy labelling)
        Mamillary nucleus (heavy labelling)
Midbrain
    Superior colliculi (heavy labelling)
Solitary tract
Reticular formation neurons

Table 1.1 (cont.)

Brainstem
: Motor neurons inc. facial and hypoglossal neurons

Choroid plexus

Stomach
: Mucosal and external muscular layers. Scattered cells in epithelium.

Duodenum

Spleen
: Heavy labelling

Kidney
: Primarily in areas of thin epithelium including thin segments of the loops of Henle and collecting tubules in the renal medulla

Thymus
: Cortex and medulla

Adrenal
: Cortex and medulla

Pancreas
: Blood vessels with both thick and thin muscular layers and cells within islets

Liver
: Light labelling

Lung
: Small and terminal bronchioles, vascular epithelium

Testes
: Ducts in epididymus (heavy labelling) and spermatocytes within epithelium. Probably not Leydig cells, presence in Sertoli cells is unknown.

Ovary
: Granulosa cells in developing follicles

Uterus
: Intense labelling of smooth muscle cells

Pituitary
: Anterior lobe (many cells)
: Intermediate lobe (a few cells)

(after Usdin et al., 1994)

Table 1.2

Distribution of VIP_1 and VIP_2 receptor mRNA in the rat

Tissue	VIP_1	VIP_2
lung	++[5]	+[3]
brain	++[1,2,5]	+[3]
stomach	-[1,2]	+[3]
colon	++[5]	+[3]
heart	-[1,2,5]	+[3]
kidney	-[5]	-[3]
skeletal muscle		-[3]
liver	+[5]	-[3]
intestine	+[5]	
jejunum	-[5]	-[3]
adrenal gland	+[1,2]/-[5]	
bone marrow	-[4]	++[4]

- - no mRNA for receptor detected
+ - mRNA for receptor present
++ - large amount of mRNA for receptor present

[1]By RT-PCR - Hashimoto et al., 1993
[2]By RT-PCR - Hosoya et al., 1993
[3]By RT-PCR - Inagaki et al., 1994
[4]By RT-PCR - Cai et al., 1997
[5]By Northern hybridisation - Ishihara et al., 1992

Table 1.3

Distribution of VIP_1 and VIP_2 and PACAP receptor mRNA in man: as assessed by RNase protection assay

Tissue	VIP_1	VIP_2	PACAP
lung	-	+	-
pancreas	-	+	-
brain	+	+	+
kidney	-	+	-
liver	+	+/-	-
skeletal muscle	-	+	-
stomach	-	+	-
heart	+	+	+
adipose tissue	+	+	+

- - no mRNA for receptor detected
+ - mRNA for receptor present
+/- - low levels of mRNA for receptor present

(after Wei & Mojsov., 1996)

Table 1.4

Cell lines expressing VIP or PACAP receptors

MIN6	a mouse insulin-secreting β cell line which has high levels of VIP_2 receptor mRNA (Inagaki et al., 1994)
HIT-T15	a hamster insulin-secreting β cell line which has moderate VIP_2 receptor mRNA levels (Inagaki et al., 1994)
RINm5F	a rat insulin-secreting β cell line which has moderate VIP_2 mRNA levels (Inagaki et al., 1994)
HT-29	a human adenocarcinoma (intestinal epithelium) cell line which expresses the VIP_1 receptor (Turner et al., 1988; Sreedharan et al., 1993)
SUP-T1	a human lymphoblast cell line which expresses the VIP_2 receptor (Svoboda et al., 1994)
NB-OK	a rat neuroblastoma cell line which expresses the PACAP receptor (Cauvin et al., 1990)
PC12	a rat phaeochromocytoma cell line which expresses the PACAP receptor (Watanabe et al., 1990)
AR4-2J	rat pancreatic acinar cell line which expresses the PACAP receptor (Buscail et al., 1990)
THP-1	a rat monocytic leukaemia cell line which expresses the PACAP receptor (Chedeville et al., 1993)
GH_3	a rat anterior pituitary tumour cell line which expresses the VIP_2 receptor (E.M. Lutz; personal communication)
GH_4C_1	a rat anterior pituitary tumour cell line which expresses the VIP_2 receptor (Rawlings et al., 1995)

Table 1.5

Distribution of PACAP Receptor mRNA in rat: as assessed by Northern Blot, Reverse Transcriptase-mediated Polymerase Chain Reaction and *in situ* Hybridisation

Tissue	
brain stem	+(NB)[1] / +(PCR)[2]
cortex	+(NB)[1] / +(PCR)[2]
olfactory bulb	++(NB)[1] / +(IS)[2]
colliculi	+(IS)[2]
hippocampus	+(NB)[1] / +(PCR)[2]
hypothalamus	+(NB)[1] / +(PCR)[2] / +(IS)[2]
thalamus	+(PCR)[2] / +(IS)[2]
occipital cortex	+(IS)[2]
cerebellum	+(IS)[2]
striatum	+(NB)[1]
pituitary	+(NB)[1] / +(PCR)[2] / +(IS)[2]
adrenal gland	+(PCR)[2] / +(IS)[2]
testes	+(PCR)[2]

\- - no mRNA for receptor detected
\+ - mRNA for receptor present
++ - large amount of mRNA for receptor present

NB - by Northern blot
PCR - by Reverse Transcriptase - Polymerase Chain Reaction
IS - by *in situ* hybridisation

([1] Morrow et al., 1993; [2] Spengler et al., 1993)

Fig. 1.4 Areas of the rat brain which express high levels of VIP_2 receptor

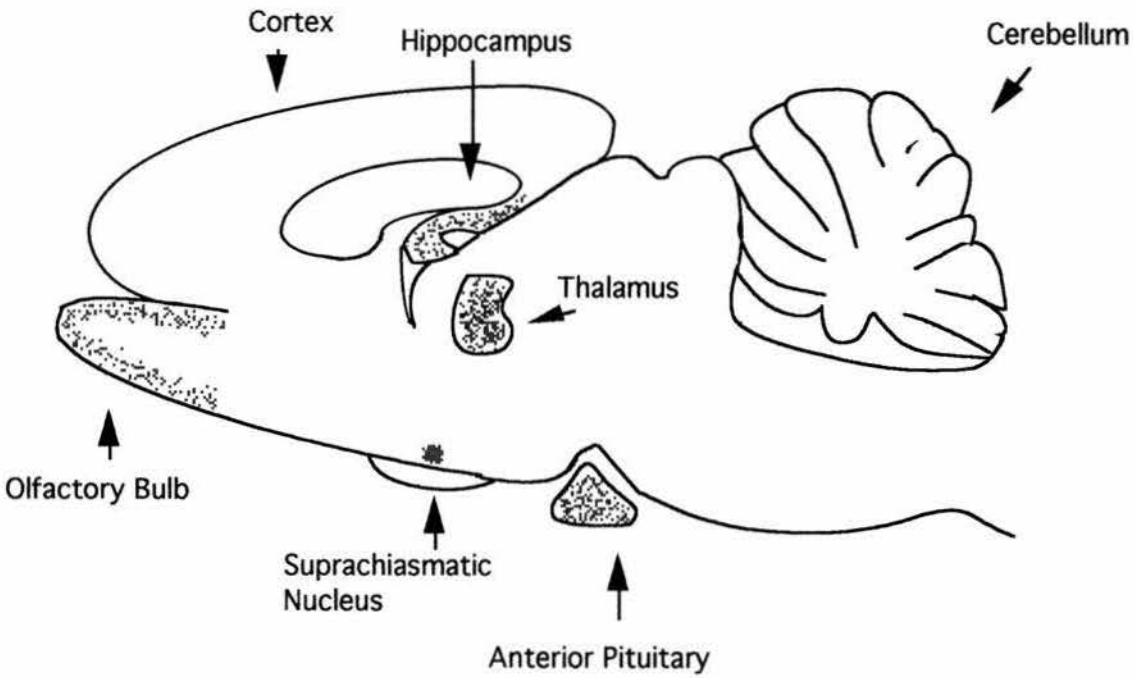
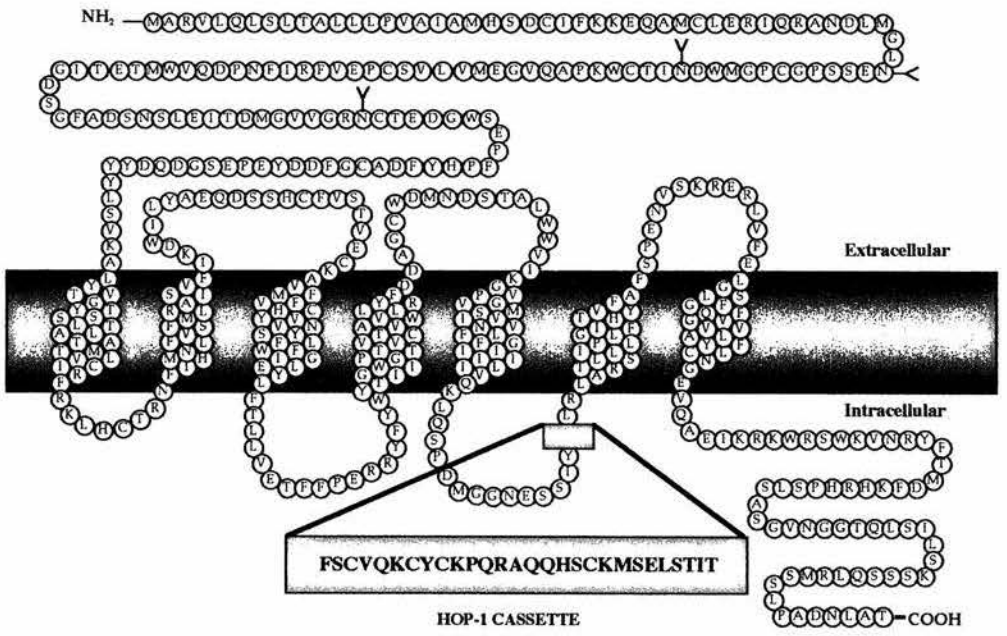


Figure 1.5 **The rat PACAP receptor with HOP-1 cassette**



The rat PACAP receptor amino acid sequence is shown. The sequence of the HOP-1 cassette insertion as found in the $PACAP_{long\ wt}$ receptor is identified in the box. The Y-shaped structures indicate the position of putative N-linked glycosylation sites.

Chapter 2

Materials & Methods

2.1 Materials

The standard laboratory chemicals were of Analar grade and the majority were obtained from BDH Chemicals Ltd., Poole, UK. Tissue culture reagents were obtained from GIBCO BRL, Life Technologies, Paisley, UK. Foetal Calf Serum was obtained from Harlan Sera-Lab, Crawley Down, UK. Tissue culture plastics were obtained from Costar UK Ltd. DEAE-dextran was from Promega, Southampton, UK. Chloroquine phosphate, ampicillin (D[-]-α-aminobenzylpenicillin), DMSO (dimethylsulfoxide), 1,1,2-trichlorotrifluoroethane, tri-n-octylamine, 3-isobutyl 1-methylxanthine (IBMX), Dowex anion exchange resin (1x8 , 200-400 mesh, H^+ form), Dowex cation exchange resin (50X8, 100-200 mesh, Na^+ form), Tween-20, Guanosine 5'-0-(2-thiodiphosphate) (GTPγS), PDBu, ionomycin, pertussis toxin, mastoparan, cholera toxin, forskolin, thapsigargin, methoxyverapamil, nifedipine, AEBSF (4-(2-Aminoethyl)benzenesulfonyl Fluoride, aprotinin, leupeptin, pepstatin, Na_3VO_4, NaF, bacitracin and soybean trypsin inhibitor were obtained from Sigma-Aldrich Co. Ltd., Poole, UK. H-89 dihydrochloride, KT-5823, KT-5720, staurosporine, Pertussis toxin B oligomer, SK&F 96365 and peptide agonists were obtained from Calbiochem-Novabiochem (UK) Ltd. Coomassie Protein Assay Reagent was pbtained from Pierce, Chester, UK. The radioligands *myo*-[2-^{3}H]inositol, L-[2,3-^{3}H]arginine and ^{125}I-PACAP-27 were from NEN-DuPont (UK) Ltd., Stevenage, UK. ^{125}I-helodermin was synthesised by John Bennie (MRC Brain Metabolism Unit, University of Edinburgh, Scotland, UK). COB-Wistar rats were obtained from Charles River UK Ltd. The COS 7 cells were a gift from Janet Allen (Glasgow University, Scotland, UK).

Primary anti-sera and control peptides for $G\alpha_s$, $G\alpha_{q/11}$ and $G\alpha_{i/o/t/z}$ were obtained from Santa Cruz Biotechnology Inc., Santa Cruz, Ca., USA. Anti-serum for $G\alpha_{t1\text{-}2}$ and control peptide were obtained from Calbiochem-Novabiochem (UK)

Ltd. PhastSystem materials were obtained from Pharmacia, UK. Immobilon transfer membranes were obtained from Millipore, UK. Enhanced ChemiLuminescence (ECL) solutions were obtained from Amersham Ltd, UK. Rabbit anti-cAMP antiserum (RIB7) was a generous gift from Dr. I. Gow, Dept. of Physiology, University of Edinburgh. Non-immune rabbit serum and all secondary anti-sera were from the Scottish Antibody Production Unit (SAPU), Carluke, Lanarkshire, UK.

The Strategene RT PCR kit and Taq polymerase were obtained from Strategene Ltd., Cambridge, UK. RNAzol B Isolation of RNA kit was obtained from Biogenesis Ltd., Poole, UK. RNaseZAP solution was from Ambion Inc., Austin, Texas, USA. The QIAGEN Plasmid Maxi Kit was obtained from QIAGEN Ltd., Crawley, West Sussex, UK.

2.2 Methods

2.2.1 Cell Culture

All cell lines were grown in a humidified atmosphere of 95% air/5% carbon dioxide at 37°C. The medium was normally changed every 3 to 4 days (every day for GH_3 cells). The cells were harvested by either trypsin digestion or a brief incubation with Hank's Buffered Saline Solution (HBSS) without calcium, magnesium or phenol red, containing 0.1% w/v EDTA. The cell medium was removed and 0.25% v/v trypsin solution in GIBCO solution A (0.4 g/l KCl, 2.2 g/l NaHCO3, 6.8 g/l NaCl, 1.0 g/l glucose, 0.005 g/l Phenol Red) was applied (1 ml per 80 cm^2 flask or 2 mls per 175 cm^2 flask) to the cell layer and then aspirated off after approx. 15 seconds. After a further 20 minutes the cells were washed off the flask's surface with medium and pelleted by centrifugation at 200 g for 5 minutes.

The cells were then resuspended in medium and distributed into new flasks (usually at a ratio of 1:3 or 1:4) or seeded into 12- or 24-well plates for assay. Alternatively the HBSS/EDTA solution was applied (1 ml per 80 cm^2 flask or 2 mls per 175 cm^2 flask) for 10 minutes before the cells were washed off as described above.

For storage purposes 0.5 ml volumes of cells at 10^6 cells/ml in medium or Foetal Calf Serum containing 7% dimethylsulfoxide (DMSO) were aliquoted into cryostat tubes and placed at -70°C overnight in a polystyrene box. The tubes were then placed into liquid nitrogen. To recover the cells from the liquid nitrogen the tubes were allowed to thaw at room temperature and then warmed in a 37°C waterbath for several minutes. A small volume of medium was added to the tube and the cells were removed and resuspended in approx. 8 mls of appropriate medium. The cells were pelleted by centrifugation at 200 g for 5 minutes before being resuspended in medium and transferred into a 25 cm^2 flask. The next day the medium was aspirated off and fresh medium added to remove traces of DMSO. The cells were then grown as normal.

i) COS 7 cells

This monkey kidney fibroblast cell line was a gift from Janet Allen (Glasgow University, Scotland, UK). These cells were maintained in Dulbecco's Modified Eagle Medium (with 580 mg/l L-Glutamine, 4500 mg/l D-Glucose, without Sodium Pyruvate) supplemented with 10% Newborn Calf Serum v/v and 100 units/ml Penicillin and 100 µg/ml Streptomycin and were passaged every 5 to 7 days when close to 100% confluency. These cells were used for transient transfection and assays of their native receptor activity.

ii) GH_3 cells

This rat anterior pituitary tumour cell line was maintained in Dulbecco's Modified Eagle Medium (with 580 mg/l L-Glutamine, 4500 mg/l D-Glucose and 110 mg/l Sodium Pyruvate) supplemented with 10% Foetal Calf serum v/v and 100 units/ml Penicillin and 100 µg/ml Streptomycin. The cells were passaged every 4 to 5 days when 50-70% confluent. The medium was changed every day.

2.2.2 Preparation of cDNA

i) Transformation of competent cells

Approx. 100 ng of cDNA was added to a 50 µl aliquot of MC1061/P3 competent cells (a strain of *Escherichia coli*) which was mixed gently and left on ice for 30 minutes. The cells were kept at 42°C for 45 seconds and placed back on ice for 10 minutes. The cells were added to 1 ml of Luria Bertani (LB)-broth (10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and placed on the rotator (New Brunswick Scientific, Edison, N.J., USA) in the 37°C incubator (Gallenkamp Plus Incunbator; Gallenkamp, Loughborough, UK) for 1 hour. 100 µl of this suspension was spread onto LB-agar plates (LB-broth + 1% bactoagar) supplemented with ampicillin at 12.5 µg/ml (Sigma) and tetracycline at 7.5 µg/ml (Gibco) to select for plasmid-containing cells. The plates were left in the 37°C incubator overnight. A distinct colony was then picked from the plate and used to inoculate a 2 ml volume of LB-broth containing ampicillin and tetracycline. The culture was incubated overnight at 37°C on the rotator. The 2 ml culture was then used to inoculate a 500 ml volume of L-broth containing ampicillin and tetracycline in a conical flask which was left on the Gallenkamp orbital incubator (Gallenkamp) overnight at 37°C.

ii) Plasmid purification

Plasmid purification was carried out using the QIAGEN Plasmid Maxi Kit (QIAGEN Ltd., Crawley, West Sussex, UK) according to the manufacturers instructions. The bacterial cells were harvested by centrifugation at 6000 x g for 15 minutes at 4°C (Sorvall GSA rotor). The supernatant was poured away and the pellet resuspended in 10 ml of Buffer P1 (resuspension buffer: 50 mM Tris Cl, pH 8.0,; 10 mM EDTA) containing 100 µg/ml RNase A solution. 10 ml of Buffer P2 (Lysis buffer: 200 mM NaOH, 1% sodium dodecyl sulphate) was added and the solution mixed gently by inversion and left to incubate at room temperature for 5 minutes. The lysis reaction causes the production of a viscous solution. 10 ml of chilled Buffer P3 (Neutralisation buffer: 3.0 M potassium acetate, pH 5.5) was added, the solution was immediately mixed by inversion and incubated on ice for 20 minutes. A viscous precipitate of genomic DNA, protein, cell debris and SDS is visible at this point. The solution was mixed again and loaded into polypropylene tubes for centrifugation in the Sorvall SS-34 rotor at 20,000 g for 30 minutes at 4°C. The supernatant containing the plasmid DNA was rapidly removed and recentrifuged for 15 minutes at 20,000 g to remove particulate matter and prevent clogging of the filters. A QIAGEN-tip 500 was equilibrated by the application of 10 ml of QBT buffer (Equilibration buffer: 750 mM NaCl, 50 mM MOPS (3-[N-Morpholino] propanesulfonic acid), pH 7.0, 15% isopropanol, 0.15% Triton X-100). The supernatant was applied to the column and washed with 2 x 30 ml of Buffer QC (Wash buffer: 1.0 M NaCl, 50 mM MOPS, pH 7.0, 15% isopropanol). The DNA was eluted with 15 ml of Buffer QF (Elution buffer: 1.25 M NaCl, 50 mM Tris HCl, pH 8.5, 15% isopropanol) and collected. The DNA was precipitated by the addition of 10.5 ml of isopropanol at room temperature, the solution was mixed and centrifuged at 15,000 g for 30 minutes at 4°C (Sorvall SS-34 rotor). The outside

of the tube was marked beforehand to identify the position of the pellet which can be difficult to see. The pellet was washed with 5 ml of room temperature 70% ethanol v/v and centrifuged at 15,000 g for 10 minutes. The last step was repeated and then the pellet was air-dried for 10 minutes before being dissolved in TE buffer (10 mM Tris HCl, pH 8.0, 1 mM EDTA) pH 8.0.

2.2.3 Transfection by the DEAE dextran method

COS 7 cells were trypsinised, or treated with EDTA as described earlier, and plated out into 80 cm^2 flasks at approx. 50% confluency for transfection the following day. The cells growth medium was replaced with prewarmed OptiMem twice (10 minutes between washes) before the transfecting medium was added to the cells. The cDNA for transfection was suspended in OptiMem (typically at 10 µg/ml) and DEAE dextran at 0.4 mg/ml (DEAE dextran stock was at 10 mg/ml in PBS and filter-sterilised), 2 mls of this solution was added to each flask and gently distributed over the cell layer. After 30 minutes incubation 3 mls of OptiMem containing 0.4 mg/ml DEAE dextran and 167 µM chloroquine phosphate (Sigma) (10 mM stock made up in PBS and filter-sterilised) was added to the solution and the flasks left to incubate for a further 3 hours. After this time the transfecting medium was aspirated off and replaced with PBS and 10% DMSO in OptiMem for 2 minutes. This medium was then aspirated off and replaced with the growth medium for transfected COS 7 cells (DMEM supplemented with 2% UltroSer G v/v (Gibco), 100 units/ml Penicillin and 100 µg/ml Streptomycin). 24 hours later the cells were trypsinised or treated with EDTA and seeded into 24- or 12-well plates for assay (two 80 cm^2 flasks per plate). For binding studies the medium in the flasks was changed and the cells were not trypsinised or EDTA treated. The cells were assayed after a further 40 hours.

2.2.4 Assay for [^{3}H]inositol phosphate production

Cells in 12-well tissue culture plates (maintained at 37°C in a 5% CO_2/95% O_2 environment) were labelled with 0.5 or 1 µCi/ml of *myo*-[2-^{3}H]inositol (NEN DuPont) for 16-18 hours in Earle's Balanced Salt Solution with 10 mM glucose, 10 mM HEPES, pH 7.4 (NaOH). The cells were then washed twice in Earle's Balanced Salt Solution with 10 mM glucose, 10 mM HEPES, 0.2% Bovine Serum Albumin (Fraction V; Sigma), pH 7.4 (NaOH) and preincubated for 10 minutes with 10 mM LiCl before agonist stimulation. Reactions were stopped by aspiration of medium and addition of 700 µl of ice-cold 1.34 M trichloroacetic acid. The wells were scraped and the solution transferred into a 1.5 ml eppendorf tube for centrifugation to pellet the precipitated protein (5 minutes, 12,000 g, 4°C), a 500 µl sample of the supernatant was then added to 50 µl of 0.1 M ethylenediaminetetraacetic acid (EDTA) and 500 µl of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. The sample was vortexed and centrifuged (5 minutes, 12,000 g, 4°C) and a 300 µl volume of the aqueous phase was added to 200 µl of 1 M $NaHCO_3$ containing universal indicator. The sample was applied to a 1 ml column of Dowex anion exchange resin (1x8 resin, formate form, 200-400 mesh; Bio-Rad) and a stepwise gradient of ammonium formate was used to elute the [^{3}H]inositol phosphates, a method previously described by Berridge et al. (1983).

The sample in the column was washed with:

1) 15 mls of UHP water

2) 5 mls of 50 mM ammonium formate

3) 10 mls of 1 M ammonium formate/0.1 M formic acid. This fraction contains the [^{3}H] inositol phosphates and was collected in a Zinsser scintillation vial (Zinsser Analytic GmbH)

4) 5 mls of 2 M ammonium formate/0.1 M formic acid

5) 15 mls of UHP water

500 µl aliquots were distributed into Pony vials (two from each collected fraction) and 4 mls of Emulsifier-Safe LSC cocktail for aqueous samples (Packard) was added to each Pony vial. The samples then shaken briefly and left overnight before being counted (4 minute count per sample, each sample counted twice) on the Beckman LS 5801 Series analyzer (Beckman).

2.2.5 Radioimmunoassay for cAMP production

Cells were washed in MEM-BSA(0.25%) and pre-incubated with 0.5 mM 3-isobutyl 1-methylxanthine (IBMX) for 15 minutes before agonist stimulation. The stimulation period was terminated by either, aspiration of the medium and addition of a 500 µl volume of ice-cold 0.1 N HCl (for intracellular cAMP measurement) or the addition of an equal volume of ice-cold 0.2 N HCl to the medium (for total cAMP measurement). The plates were then frozen at -70°C. After thawing, the cells were homogenised by trituration. Duplicate 50 µl aliquots were taken from each well and assayed for cAMP content by radioimmunoassay.

All solutions were made up in 50 mM sodium acetate buffer (pH 6.0), 0.1% BSA (fraction V), 0.1% sodium azide and the assays carried out in polypropylene microrack tubes (1.2 ml, Alpha Labs). RIB7 cAMP antiserum at a final concentration of 1:166,000 was used as a primary antibody, ^{125}I-cAMP at approx. 167 KBq per tube was added and the tubes were mixed by Vibrax and left at 4°C overnight. Donkey anti-rabbit IgG (at a titre of 1:400) was used as a secondary antibody and non-immune rabbit serum was also added at a titre of 1:40 for a final assay volume of 275 µl. The tubes were mixed and incubated at 4°C for 3 hours. 700 µl of ice-cold PEG-8000 (8.7%) in 0.1 M sodium phosphate buffer (pH 7.7) was then added to each tube in order to aid the precipitation of the ^{125}I-

cAMP/antibody complex and the tubes were spun at 1,500 g for 25 minutes at 4°C. The supernatant was then aspirated off and the gamma radiation remaining in the pellet, and thereby the cAMP content, was determined using the Cobra Autogamma counter (Packard) and the associated software. The specific binding was typically 40% of the total applied counts and the non-specific binding typically comprised less than 10% of the specific binding. The assay included a cAMP standard curve running from 1 to 512 nM cAMP. The cAMP concentration of the unknowns were determined by interpolation of the % Bound counts and the log of the cAMP concentration:

$$\% \text{ Bound counts} = \frac{\text{unknown - non-specific binding}}{\text{total binding - non-specific binding}} \times 100$$

2.2.6 Assay for [^{3}H]citrulline production

Cells in 12-well tissue culture plates (maintained at 37°C in a 5% CO_2/95% O_2 environment). The normal growth medium was replaced with Earle's Balanced Salt Solution with 10 mM glucose, 10 mM HEPES, pH 7.4 (NaOH) for 16-18 hours. 3 µCi/ml of L-[2,3-^{3}H]arginine (NEN DuPont) was added to the medium for 30-60 minutes. The medium containing label was then aspirated off and replaced with fresh medium. Agonist was then added for a 10 minute incubation period. Reactions were stopped by aspiration of the medium and addition of 700 µl of ice-cold 1.34 M trichloroacetic acid. The wells were scraped and the solution transferred into a 1.5 ml eppendorf tube for centrifugation to pellet the precipitated protein (5 minutes, 12,000 g, 4°C), a 500 µl sample of the supernatant was then added to 50 µl of 0.1 M ethylenediaminetetraacetic acid (EDTA) and 500 µl of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-n-octylamine. The sample was vortexed and centrifuged (5 minutes, 12,000 g, 4°C) and a 300 µl volume of the

aqueous phase was added to 1.75 mls of 20 mM HEPES pH 6.0 with 1 mM citrulline.

The sample was then applied to a 1 ml Dowex cation exchange resin column (50X8, 100-200 mesh, Na form; Sigma). The column was prepared by washing with 4 mls 2 M Na Acetate, 0.1 M NaOH. The sample was applied to the column and the 'run-through' was collected in a Zinsser scintillation vial, a further fraction was eluted with 4 mls of UHP H_2O and also collected in a Zinsser scintillation vial. The column was then washed with 8 mls 20 mM Na HEPES pH 6.0. Duplicate 500 µl aliquots were taken from each of the collected fractions. and transferred into Pony vials 4 mls of Emulsifier-Safe LSC cocktail for aqueous samples (Packard) was added to each. The samples then shaken briefly and left overnight before being counted (4 minute count per sample, each sample counted twice) on the Beckman LS 5801 Series analyzer (Beckman).

2.2.7 Ligand-binding studies

i) Preparation of membranes

A confluent flask of cells (80 cm^2) was placed on ice and the medium discarded. The cell layer was gently washed with 10 mls of ice-cold EBSS. 1 ml of Buffer A (50 mM Tris HCl pH 7.4, 1 mM EGTA (ethyleneglycol-bis(b-aminoethyl ether)-N,N,N',N'-tetraacetic acid), 1 mM AEBSF (4-(2-Aminoethyl)benzenesulfonyl Fluoride, 2 µg/ml aprotinin, 4 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM Na_3VO_4, 1 mM NaF, 50 µg.ml soybean trypsin inhibitor) was added to the cell layer and the cells scraped and transferred into a clean tube on ice. Another 1 ml volume of Buffer A was added to the flask and the process repeated. The cells were then homogenised using the Ystral cell homogeniser (Scientific Industries International, Loughborough, UK) at setting 3 for 30 seconds. A sample of homogenate was taken

for protein determination. The homogenate was aliquoted into 1.5 ml eppendorf tubes and centrifuged at 1,000 g for 5 minutes at 4°C. The supernatant was transferred into fresh tubes leaving the pellet of intact cells, nuclei etc. behind. The supernatant was centrifuged at 12,000 g for 30 minutes at 4°C. The pellet formed contained the membranes, the supernatant was discarded. A 1 ml syringe with a 20G$1^1/_2$ 40/9 Yale Microlance needle was used to resuspend the pellet from 1 flask in 1.0 ml Buffer A, a sample of this was taken for protein determination. The suspension was then recentrifuged as previously and the membranes resuspended in 1-2 ml of assay buffer (50 mM Tris HCl, pH 7.4, bacitracin 0.5 mg/ml, AEBSF 2 µg/ml, BSA 1%).

ii) Homologous and heterologous displacement of ligand from membranes

The assay was normally carried out at 37°C in a shaking water bath. The assay volume was 500 µl in a screw-top eppendorf tube. 300 µl of assay buffer, 50 µl of 10X peptide (normally helodermin, VIP or PACAP-27) and 50 µl of ^{125}I-helodermin or ^{125}I-PACAP-27 was added to each tube. A high dose of displacing peptide (e.g. 10 µM) was used to determine non-specific binding The reaction was started by the rapid addition of 100 µl of membrane suspension using an Eppendorf repeater pipette. The samples were stimulated for 10-15 minutes. The reactions were stopped by centrifugation at 12,000 g for 30 minutes at 4°C. The supernatant was then aspirated off, 500 µl of assay buffer was added to each tube and the samples were recentrifuged as above but for 15 minutes. The supernatant from the samples was then aspirated off again and the bottom of the eppendorf tube (which contained the pellet) was clipped into a disposable tube for the counter. The gamma radiation remaining in the pellet and therefore the amount of membrane-associated iodinated ligand in the samples, was determined using the Cobra Autogamma counter (Packard) and the associated software.

iii) Homologous displacement of ligand from whole cells at 0°C.

Transfected cells in 12-well plates were washed twice and incubated for 60 minutes on ice in Medium 199 containing 0.2% BSA and [^{125}I]PACAP-27 or [^{125}I]VIP in the absence or presence of increasing concentrations of unlabelled PACAP-27 or VIP. A high dose of displacing peptide (e.g. 10 μM) was used to determine non-specific binding. Unbound radioactivity was removed by washing cells three times with EBSS containing 0.1% BSA. Bound radioactivity was removed by an acid wash procedure, a 5 minute incubation with 0.2 M acetic acid/0.5 M NaCl, and the gamma radiation measured using the Cobra Autogamma counter and the associated software.

iv) Homologous displacement of ligand from whole cells at 37°C.

Transfected cells in 12 -well plates were washed twice and incubated at 37°C in a waterbath for 10 mins in Medium 199 containing 0.2% BSA, 30 μg/ml bacitracin and 1 μg/ml aprotinin with [^{125}I]VIP or [^{125}I]PACAP-27 in the absence or presence of increasing concentrations of unlabelled VIP or PACAP-27. A high dose of displacing peptide (e.g. 10 μM) was used to determine non-specific binding. Unbound radioactivity was removed by washing cells three times with Earles Balanced Salt solution (EBSS) containing 0.1% BSA. Bound radioactivity was removed by acid wash (0.2M glacial acetic acid/0.5 M NaCl) for 5 mins on ice. The amount of internalised radioactivity was also determined by solubilising the cell layer by treatment with 0.5 M NaOH overnight. Gamma radiation in the samples was measured by the Cobra Autogamma counter and the associated software.

v) GTPγS modulation of ligand-binding

This procedure is identical to the membrane ligand binding protocol above except that the effects of increasing concentrations of GTPγS (Guanosine 5'-0-(2-

thiodiphosphate): Sigma) on the binding of an iodinated ligand (^{125}I-helodermin) are investigated as opposed to the effects of increasing concentrations of unlabelled peptide. 10X GTPγS replaced the 10X peptide in the assay. A high dose of helodermin was used to determine non-specific binding.

2.2.8 Protein Assay

Protein concentration was determined using the Coomassie Protein Assay Reagent (Pierce, Chester, UK). A standard curve was constructed using a 2 mg/ml albumin standard solution (Pierce, USA) covering the range from 75 to 1500 µg/ml. Duplicate 10 µl volumes of sample were added to the wells in a 96-well microtiter plate, 300 µl of Coomassie reagent was then added to each well and the samples left for 30 to 45 minutes. The absorbance at 595 nm in each well was then determined using the Microplate Biokinetics Reader EL 312e (BIO TEK Instruments, Vermont, USA) running the KC Jr. application software.

2.2.9 Animals

COB-Wistar rats obtained from Charles River UK Ltd., or bred in this department from rats originally obtained from this source, were maintained at a constant temperature of 22°C under controlled lighting, which was on from 0500 to 1900 hours each day. The rats had free access to tap water and food pellets. The rats were killed by cervical dislocation and the brains were rapidly dissected out and the appropriate tissue removed for homogenisation.

2.2.10 Cerebral Microvessel Preparation

The brain was removed from 4 to 8 male COB-Wistar rats. The cortex was rapidly separated from the other brain tissue and placed into phosphate-buffered saline (PBS) pH 7.4. Ice-cold solutions are used at all times through this procedure.

The tissue was weighed and then homogenised with 20 mls PBS pH 7.4 (per 4 rats) in a p.t.f.e./glass homogeniser (30 ml capacity, 25 mm bore; Philip Harris) 12 passes by hand then 30 seconds at slow speed on the variable speed stirrer (Griffin & George, UK). The homogenate was distributed into 2 centrifuge tubes and the volumes made up to 12 mls PBS pH 7.4 per tube. The homogenate was spun in the Sorvall RC-3B refrigerated centrifuge (DuPont, UK): 420 g, 4°C, 10 minutes. The supernatant was aspirated off leaving a large pellet. 9 mls of PBS pH 7.4/15% dextran (580K) w/v was added to the pellet which was resuspended by vortexing. The solution was spun again in the Sorvall centrifuge: 950 g, 4°C, 20 minutes. The suspension now had a large floating layer of pink material, this was aspirated off leaving a pale dispersed pellet in the bottom of the centrifuge tube. 3 mls of PBS pH 7.4 was added to the tube which was then vortexed. The suspension was poured over a 100 µm mesh-opening net filter, 25 mm diameter in a 2 piece glass filter holder (Millipore, UK). The holder and filter were pretreated with RNaseZAP (Ambion, Inc., USA) and washed with UHP H_2O and 24 mls of PBS pH 7.4 before the suspension was added. The suspension on the filter was washed with 36 mls PBS pH 7.4 the filter with the trapped microvessels was then removed and placed in the -70°C freezer. Examination of the filter at X200 magnification confirmed the presence of bloodvessels on the mesh.

2.2.11 RNA extraction

RNA extraction was carried out using the RNAzol B Isolation of RNA kit (Biogenesis Ltd., Poole, UK) which functions by promoting the interaction of RNA with guanidinium and water ions and inhibiting the hydrophilic interactions of DNA and proteins thereby significantly purifying the sample through a simple phase separation step.

The microvessel/filter preparation was homogenised in an RNaseZAP-treated, UHP H_2O rinsed, p.t.f.e./glass homogeniser (Millipore, UK) containing 1 ml RNAzol B. 100 µl of chloroform was added to the sample in an RNase-free eppendorf and the solution was shaken vigorously for 15 seconds by hand. The sample was then centrifuged at 12,000 g for 15 minutes at 4°C. The aqueous upper phase is equivalent to 50% of the RNAzol volume plus a volume of the tissue used for homogenisation. 800 µl of the upper phase was transferred to a fresh tube and an equal volume of isopropanol was added to it. The sample was stored at 4°C overnight before being centrifuged at 12,000 g for 15 minutes at 4°C. After this step the pellet was obvious as a smear up the side of the eppendorf tube. A drawn pipette was used to aspirate off the supernatant and 1 ml of 80% ethanol was added, the tube was shaken vigorously to dislodge the pellet and the sample was centrifuged at 12,000 g for 5 minutes at 4°C. A drawn pipette was used to aspirate off the supernatant and the tube was left open on it's side on the bench for 10 minutes, inside an open plastic bag, to allow the ethanol to evaporate from the pellet. The pellet was then solubilised in 100 µl of tissue culture water (Gibco, UK), 10 µl 5M NaCl (Ambion Inc., USA)and then reprecipitated by the addition of 200 µl of ethanol. The solution was left at -20°C for 1 hour. The sample was then centrifuged at 12,000 g for 20 minutes at 4°C. The supernatant was aspirated off using a drawn pipette and the pellet was washed once more in 1 ml of 80% ethanol and the sample centrifuged at 12,000 g for 5 minutes at 4°C. The pellet was allowed to dry on the bench as previously for 10 minutes and then placed in -70°C freezer.

2.2.12 Reverse Transcriptase-Mediated Polymerase Chain Reaction

Fragments from the total RNA extracted from tissue were amplified using the Stratagene RT-PCR kit according to the manufacturers instructions.

i) 1st Strand Reaction

5 - 10 µg of total RNA in DEPC-treated water in a final volume of 38 µl was used, 300 ng of random primers was added in 3 µl and the solution mixed gently. All reactions were carried out in GeneAmp).5 ml thin-walled reaction tubes (Perkin-Elmer). The mixture was then incubated at 65°C for 5 minutes in the Omn-E Thermal Cycler (Hybaid Ltd., Middlesex, UK)) before being cooled to room temperature over 15 minutes to allow annealing of the primers to the RNA. 5 µl of 10X first strand buffer was then added to each tube, 40 U of RNase Block Ribonuclease Inhibitor in 1 µl, 2 µl of 100 mM dNTPs (final conc. 1 mM each dNTP) and 1 µl of Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (50 U). The mixture was incubated at 37°C for 1 hour and 90°C for 5 minutes. The completed reactions were then placed on ice.

ii) Polymerase Chain Reaction

1-5 µl of 1st strand reaction (made up to 5 µl using DEPC-treated water) was transferred into a fresh tube and 88 µl of a "master mix" was added. The "master mix" consisted of 10 µl of 10X *Taq* DNA polymerase buffer, 0.8 µl 100 mM dNTPs and 77.2 µl of DEPC-treated water for a final volume of 88 µl per tube. 15 pM of each primer in 1 µl is added to each tube (total of 2 µl primers per tube). Mineral oil was then added to the tube to prevent evaporation. The tubes were place in the thermal cycler and heated to 95°C for 5 minutes, 60°C for 5 minutes and 72°C for 3 minutes. The samples were kept at a holding temperature of 72°C for addition of the *Taq* DNA polymerase (Stratagene, Cambridge, UK) which was added as a 5 µl volume of 0.4 U/µl. The PCR cycling was normally carried out for 35 cycles of 1 minute at 94°C, 1 minute at 60°C and 1 minute at 72°C. The samples were then kept at 72°C for 30 minutes before being returned to room temperature.

iii) Agarose gel electrophoresis of DNA

A 100 ml 0.7% (w/v) agarose gel (Sigma, UK) containing 0.1 µg/ml ethidium bromide (Sigma, UK) was made up in 1X Tris-Acetate-EDTA buffer (4.84 g Tris base; 1.142 ml glacial acetic acid; 2 ml 0.5 M EDTA per litre: 10X from Sigma, UK). The 110 mm x 140 mm gel was run on the BRL H5 horizontal submerged gel electrophoresis system (BRL, MD., USA) using the LKB 2197 power supply (Bromma). The gel was submerged in 800 ml of 1X TAE buffer, 10 µl of cDNA was added to 4 µl of loading buffer (0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol, 1 mM EDTA, 30% glycerol) for addition to the gel. The gel was run for approx. 1 hour 45 minutes at 100 volts, 60-80 milliamps. Boehringer Mannheim molecular weight markers VI and VII were used (Boehringer Mannheim GmbH, Mannheim, Germany) at 3 µl per lane.

The DNA bands were visualised using the Chromato-vue transilluminator model TM-15 (UVP Inc., Ca., USA). The gels were photographed using a Polaroid Cu-5 camera and Polaroid 667 film (Sigma).

2.2.13 Immunoprecipitation

3 mls of Buffer B (20 mM HEPES, 1 mM AEBSF, 2 µg/ml aprotinin, 4 µg/ml leupeptin, 2 µg/ml pepstatin, 1 mM sodium orthovanadate, 1 mM sodium flouride, 50 µg/ml soybean tryspin inhibitor).was added to the cell layer of a 75cm^2 flask and the cell layer scraped and transferred into a clean tube on ice. The cells were then homogenised using the Ystral cell homogeniser at setting 3 for 30 seconds. The homogenate was centrifuged at 12,000 g for 30 minutes. The pellet was resuspended in Buffer B and recentrifuged at 12,000 g for 30 minutes. The pellet was then solubilised in 0.7 ml of Buffer B with 5 mM CHAPS, 0.1% cholate and1 M sodium chloride and left 'rolling' at 4°C for 30 minutes. The salt concentration was

then reduced by the addition of an equal volume of Buffer B with 5 mM CHAPS and 0.1% cholate. 15 µl of Protein G-sepharose suspension (1:100: Sigma) was added to the tube which was left 'rolling' at 4°C for 15 minutes. The solution was centrifuged at 12,000 g for 30 minutes. The supernatant was removed and primary antibody (1:100, 'mHA' anti-HA; Boehringer-Mannheim) was added. The tube was left 'rolling' overnight at 4°C. 25 µl of Protein G-sepharose suspension (1:100: Sigma) was added to the tube and left 'rolling' at 4°C for 4 hours. The suspension was centrifuged at 12,000 g for 15 minutes. The supernatant was removed and the pellet was resuspended in 'Novamok' (20 mM HEPES, 5 mM CHAPS, 0.1% cholate) and centrifuged at 12,000 g for 15 minutes. The supernatant was discarded and the pellet resuspended in 2X Laemmli Buffer (2% SDS, 50 mM Tris, 5% MeOH). The sample was boiled for 5 minutes before undergoing SDS -PAGE Electrophoresis.

2.2.14 Western Blotting

Heat thawed samples for 5 minutes under hot water tap (approx. 50°C) and centrifuge for 2 minutes at room temperature in Eppendorf centrifuge to remove particulate material.

i) SDS -PAGE Electrophoresis

The 7.5% homogeneous polyacrylamide gel pre-cast onto a rigid polyester film (PhastGel Homogeneous 7.5, Pharmacia) was removed from its wrapping and carefully placed onto 80 µl of UHP H_2O on the gel bed of the PhastSystem Separation and Control unit (Pharmacia Biotech AB, Uppsala, Sweden) in such a manner as to exclude air bubbles. The gels have a 13 mm stacking zone and 32 mm separation zone (dimensions; 43 x 50 x 0.45 mm). The PhastGel SDS buffer strips were then positioned in the buffer strip holder. The samples were loaded (4 µl)

onto the sample strip holder in a humidified chamber and the holder was positioned in the apparatus. The separation program was then started to run for 60 Vh (approx. 30 mins).

ii) Electroblotting

Immobilon transfer membranes (polyvinyldifluoride, 10 cm x 10 cm, 0.45 μM pore size; Millipore) were cut to size (5 cm x 5 cm) and marked for identification and orientation. The filters were immersed in methanol and then rinsed in UHP H_2O briefly 5 times. The filters were then shaken for 2 x 5 minutes in UHP H_2O and in blot buffer (25 mM Tris, 192 mM glycine, 20% methanol, pH 8.3) for 3 x 5 minutes using the rotary shaker.

After the gel had run it was removed from its backing using Pharmacias clamp and wire apparatus. The immobilon was placed over the separation zone of the gel and the gel was removed from its backing whilst remaining in contact with the immobilon. 3 pieces of PhastTransfer filter paper (50 mm x 50 mm) were soaked in blot buffer and placed on the anode of the PhastSystem Development Unit, 6 more drops of blot buffer were applied to the filters and the immobilon placed on top of them. Another 3 filters were then soaked and placed on top of the immobilon/gel. The cathode was then placed on top of the filters and pressed into place gently. The blot was run for 45 minutes.

iii) Immunostaining

The blot was given 5 quick rinses in UHP H_2O before being given 4 x 4 minute washes on the shaker. The blot was then stained for 5 minutes in 0.1% coomassie blue-R250, 50% methanol before being given 2 or more 1 minute washes in destain (50% methanol, 10% acetic acid) until the protein bands were clearly visible against a clear or pale background. Several 1 minute washes in UHP H_2O

were then carried out to stop the destaining process and remove residual methanol. The blot was then marked and the lanes cut up as appropriate. The blot was washed two or more times for 2 minutes in methanol until the bands were no longer visible. A further 3 x 4 minute washes in methanol were carried out before the blot was given 5 x 5 minute washes in UHP H_2O to remove residual methanol.

The blot was incubated overnight at 4°C in either 5% BSA (w/v)/PBS or 5% dried milk. The blot was given 2 quick rinses and 5 x 5 minute washes in PBS/0.1% Polyoxyethylenesorbitan monolaurate (Tween-20;Sigma). The blot was incubated overnight with the primary antibody in 0.25% BSA/PBS/0.05% Tween-20:

1) $G_{\alpha\ i1\text{-}2}$ (Calbiochem, Nottingham, UK) - anti-$G_{\alpha\ i1}$ and $G_{\alpha\ i2}$ (C terminal, 345-354 and 346-355) rabbit polyclonal IgG. Supplied in 100 mM potassium phosphate, 140 mM NaCl, pH 7.5. Original titer 1:8,000. IgG added as 2 µl/ml, control peptide as 20 µl/ml.

2) $G_{\alpha\ q/11}$ (Santa Cruz) - anti-$G_{\alpha\ q/11}$ ($G_{\alpha q}$ 341 -349, mouse) rabbit polyclonal IgG. 100 µg of IgG in 1 ml PBS/0.1% sodium azide /0.2% gelatin. Control peptide 100 µg in 0.5 ml PBS containing 0.1% sodium azide/100 µg BSA. IgG added as 5 µl/ml, control peptide as 25 µl/ml.

3) $G_{\alpha\ i/o/t/z}$ (Santa Cruz) - anti-$G_{\alpha\ i/o/t/z}$ ($G_{\alpha\ z}$ C terminus, 325-344, rat) rabbit polyclonal IgG. 100 µg of IgG in 1 ml PBS/0.1% sodium azide/0.2% gelatin. Control peptide100 µg in 0.5 ml PBS containing 0.1% sodium azide/100 µg BSA. IgG added as 5 µl/ml, control peptide as 25 µl/ml.

An appropriate blocking peptide was mixed with the primary antibody for use as a control.

The blot was given 2 quick rinses and 5 x 5 minute washes in 0.25% BSA/PBS/0.1% Tween-20 (Sigma) and incubated for 1 hour with horseradish peroxidase (HRP)-labelled anti-rabbit IgG (Scottish Antibody Production Unit) at a 1:5000 dilution in 0.25% BSA/PBS/0.05% Tween-20. The blot was then rinsed as previously.

In the darkroom the blot was incubated for 1 minute in 2.5 ml of a 1:1 mixture of Enhanced ChemiLuminescence (ECL) 1:ECL2 solutions (Amersham). Excess liquid was removed from the blot by touching the edge with a mediwipe tissue. The blot was then sealed in clingfilm and exposed against ECL film in an X-ray cassette for between 30 seconds and 5 minutes. The film was developed by incubation for 4 minutes in Phenisol developer, rinsing in water, and a 1 minute incubation in Hypam fixer followed by a minimum of 2 minutes in running water.

Chapter 3

Signalling characteristics of the wild-type VIP_2 receptor

3.1 Introduction

In order to study the expression and signalling characteristics of the cloned rat VIP_2 receptor in contrast with $PACAP_{short}$ and $PACAP_{long}$ receptors, a heterologous expression system was used. The receptors were mainly transiently expressed in a Green Monkey kidney fibroblast cell line, COS 7 cells. The primary objective of this study was to investigate VIP_2 receptor-mediated signalling. However, the signalling characteristics of the closely related PACAP receptors provide a useful comparison for VIP_2 receptor-mediated signalling and because of the high degree of sequence homology between the receptors, the comparison could provide indications of the motifs and domains important for any differential aspects of their signalling behaviour. This approach was also exploited in the creation of the chimaeric receptors used in the experiments described in Chapter 4.

The rat VIP_2 receptor was cloned by Lutz et al. (1993) from a rat olfactory bulb cDNA library. When expressed in COS 7 cells this receptor was found to be coupled to cAMP production. The order of potency of ligands for cAMP production mediated by this receptor was VIP~PACAP-38~PACAP-27~helodermin>PHI~rGHRH. Two isoforms of the rat PACAP receptor were cloned from a rat olfactory bulb cDNA library by Morrow et al. (1993), one isoform contained an extra 84 bp segment in the putative intracellular loop 3(IC3), this receptor was designated $PACAP_{long}$ in this study, the other isoform being $PACAP_{short}$. When transiently expressed in COS 7 cells these receptors were also found to be coupled to cAMP production. The order of potency of ligands for this stimulation was PACAP-38~PACAP-27>VIP>PHI. All known members of the secretin/calcitonin/PTH/PTHrP receptor family are coupled to cAMP production (Segre & Goldring, 1993). The rat PACAP receptors were additionally shown to be capable of stimulating PI hydrolysis and the mobilisation of intracellular Ca^{2+} in

tissue (Gottschall et al., 1991; Deutsch & Sun., 1992; Tatsuno et al., 1992). Spengler et al. (1993) identified further splice variants of the rat PACAP receptor and demonstrated the ability of the PACAP receptor to stimulate PLC when expressed in LLC PK1 cells.

Although there were indications that the agonist-activation of VIP receptors was capable of stimulating PI hydrolysis and Ca^{2+}-release from intracellular stores and activating PKC (Audigier et al., 1986; Malhotra et al., 1988; Weill et al., 1989; Russell et al., 1990; Fatatis et al., 1994) there was no direct evidence of PLC stimulation by VIP_1 or VIP_2 receptors.

The VIP receptor in rat lung was found to be coupled to both G_s (Kermode et al., 1992) and G_{i3} (Diehl et al., 1996), as determined by cross-linking studies, whereas Murthy & Makhlouf (1994) reported the coupling of a VIP receptor in dispersed gastric smooth muscle cells to $G_{i1/2}$.

VIP receptors have been implicated in, amongst other things, the control of cerebral energy metabolism and cerebral blood flow, neuronal survival (Gozes et al., 1989; Arimura, 1992), insulin release from the pancreas (Yada et al., 1994; Straub et al., 1996), melatonin synthesis and release from the pineal (Spessert, 1993; Simmoneaux et al., 1993), catecholamine synthesis and release from the adrenal medulla (Wakade et al., 1991; Watanabe et al., 1995), smooth muscle relaxation, stimulation of electrolyte secretion and vasodilation in the peripheral nervous system (Gozes & Brenneman, 1989; Christophe, 1993). Despite the obvious physiological importance of VIP-evoked and VIP receptor-mediated signalling events, the specific signalling mechanisms utilised by the VIP receptors were poorly understood.

3.2 Specific Methodology

Expression of receptors - COS 7 cells were transiently transfected with cDNA encoding the rat VIP_1, VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors and the C-terminally haemagluttinin(HA)-tagged human VIP_2 receptor as described in sections 2.2.1, 2.2.2 and 2.2.3. A C-terminally myc-tagged human VIP_2 receptor was created by Dr. Eve Lutz in this laboratory, Dr. T. MacDonald exchanged the myc tag for an HA tag and that construct was used here.

Ligand-binding - Ligand binding on whole cells at 0°C for 60 minutes using iodinated ligand was carried out to determine the expression level of the receptors (see section 2.2.7). GTPγS modulation of ligand-binding was investigated using iodinated ligand on membrane preparations from transfected COS 7 cells (see section 2.2.7).

Protein assay - Coomassie protein assay reagent was used to determine the protein level in samples (see section 2.2.8).

Second messenger assays - Intracellular cAMP production over a 10 minute stimulation period was measured in whole cells by radioimmunoassay as described in Section 2.2.5.

[^{3}H]inositol phosphate production was used as a measure of PLC activity. The cells were stimulated for 60 minutes with agonist before separation of [^{3}H]inositol phosphates by anion exchange chromatography as described in Section 2.2.4.

Immunoprecipitation - Immunoprecipitation of the HA-tagged human VIP_2 receptor expressed in COS 7 cells was accomplished with the use of a specific antibody to the haemagluttinin sequence expressed at the C-terminal end of the receptor (see section 2.2.13).

Western blotting - Western blotting for specific G protein isoforms associated with the immunoprecipitated human VIP_2 receptor was carried out after using the Phast-system after SDS-PAGE of the immunoprecipitate, as described in section 2.2.14.

Data analysis - Curve fitting was performed by the non-linear curve-fitting programme, P-fit (Elsevier Biosoft, Cambridge).

3.3 Results

3.3.1 Ligand-binding studies to monitor expression levels of the rat VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors and their affinities for PACAP-27

Homologous displacement of [^{125}I]PACAP-27 from whole COS 7 cells (see 'Materials & Methods' section 2.2.7) was used to give an estimate of the receptor binding capacity of the system (B_{max}) and the affinities of the receptors for PACAP-27 (IC_{50}) (see Figure 3.1), using the method of Swillens (Swillens, 1992). Binding was performed on whole cells at 0°C for 60 minutes (see Figure 3.1). PACAP-27 was used as opposed to PACAP-38 because it was the only PACAP and VIP_2 receptor agonist commercially available as a radioligand. The $PACAP_{long}$ receptor had a B_{max} value of 520±52 fmol/10^5 COS 7 cells (see Table 3.1) and an IC_{50} of 31±3 nM. The $PACAP_{short}$ receptor displayed a B_{max} of 494±37 fmol/10^5 cells and an IC_{50} of 30±1 nM. The VIP_2 receptor had a B_{max} of 59±5 fmol/10^5 cells and an IC_{50} value of 19±1 nM.

The expression levels of the PACAP receptors were therefore very similar as was their affinity for PACAP-27. The VIP_2 receptor was however expressed at a lower level than the PACAP receptors (approximately 11% of the PACAP receptor value) and had a slightly higher affinity for PACAP-27.

3.3.2 Agonist-evoked cAMP production mediated by the rat VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors

Concentration-response curves for VIP- and PACAP-38-evoked cAMP production were constructed for the VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors. Figure 3.2 shows a typical concentration-response curve for VIP-evoked cAMP production mediated by the VIP_2 receptor transiently expressed in COS 7 cells. The EC_{50}s for PACAP-38 evoked cAMP production were similar for the VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors (in the range 0.5-0.9 nM: see Table 3.1). Whereas VIP and PACAP-38 had similar potencies and maxima for cAMP production mediated by the VIP_2 receptor (0.3±0.1 nM and 7.8±0.5-fold of basal control for PACAP-38 and 0.9±0.1 nM and 8.2±0.1-fold of basal control for VIP). VIP was very much less potent than PACAP-38 at stimulating cAMP production mediated by the $PACAP_{short}$ and $PACAP_{long}$ receptors (EC_{50} values of 28.6±0.2 and 38.6±3.8 nM respectively) and the maxima were also reduced (from 22.4±1.1 to 12.1±0.5-fold of basal control for $PACAP_{long}$ and 19.8±1.4 to 15.7±0.2-fold of basal control for the $PACAP_{short}$ receptors).

3.3.3 Agonist-evoked [^{3}H]inositol phosphate production mediated by the rat VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors

Concentration-response curves for VIP- and PACAP-38-evoked VIP_2 receptor-mediated [^{3}H]IP production (see Figure 3.3) were therefore carried out. These curves showed clear agonist-evoked concentration-dependent increases in [^{3}H]IP production mediated by this receptor when transiently expressed in COS 7 cells (see Table 3.1). The VIP_2 receptor displayed an EC_{50} for VIP-evoked [^{3}H]IP production of 45.0±6.6 nM and a maximum stimulation of 2.4±0.1 fold of basal control; for PACAP-38 the EC_{50} was 36.9±8.1 nM and the maximum stimulation 3.0±0.3 fold of basal control. Concentration-response curves for VIP- (see Table

3.1) and PACAP-38-evoked $PACAP_{short}$ and $PACAP_{long}$ receptor-mediated [^{3}H]IP production were also carried out (see Fig. 3.4). The EC_{50}s for PACAP-38-evoked [^{3}H]IP production were 10.6±0.6 nM and 17.6±0.6 nM, respectively and the maximum stimulation values were 9.8±0.7 and 11.1±0.3 fold of basal control respectively for the $PACAP_{short}$ and $PACAP_{long}$ receptors. No VIP-evoked [^{3}H]IP production mediated by the $PACAP_{short}$ and $PACAP_{long}$ receptors was detected at concentrations up to 3 μM.

This represents the first direct evidence of VIP_2 receptor -mediated stimulation of [^{3}H]IP production. PACAP-38 was approximately 2.5 times more potent in eliciting [^{3}H]IP production at the PACAP receptor than at the VIP_2 receptor. The PACAP receptor also mediated a higher fold stimulation of [^{3}H]IP production than the VIP_2 receptor. VIP and PACAP-38 were equipotent for [^{3}H]IP production mediated by the VIP_2 receptor whereas VIP was ineffective at the PACAP receptor.

3.3.4 Time-courses for agonist-evoked cAMP and [^{3}H]inositol phosphate production at the VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors

In order to determine whether the VIP_2 or PACAP receptor-mediated stimulation of [^{3}H]IP production or cAMP production was subject to desensitisation, the accumulation of [^{3}H]IP was measured for up to 80 minutes and cAMP for up to 120 minutes. The 1 μM VIP-evoked accumulation of [^{3}H]IP mediated by the VIP_2 receptor was linear over the measured time-period; there was no evidence of desensitisation of this response (see Figure 3.5), although the dose of agonist used may affect this response (it is also the case that earlier time points would be advantageous in these time courses since an early peak of IP production might be expected and this would not be detected with a first time point at 10 minutes). The 300 nM PACAP-38-evoked response mediated by the receptor (see

Figure 3.6) was also linear over the measured time-period and showed no evidence of desensitisation. The maximum responses mediated by the VIP_2 and $PACAP_{long}$ receptors were 2.01±0.05 and 7.31±1.04 fold of basal control. The 100 nM PACAP-38-evoked stimulation of cAMP production mediated by both the VIP_2 and PACAP receptors was clearly subject to desensitisation (see Figure 3.7). Responses mediated by the VIP_2 and PACAP receptors involved the rapid accumulation of intracellular cAMP until a plateau phase was reached after approximately 15 minutes. At their respective 15 minute time points the VIP_2 receptor had caused a 13.7±0.4 fold of basal control increase in intracellular cAMP, the $PACAP_{short}$ receptor mediated a 27.1±0.9 fold of basal control increase and the $PACAP_{long}$ receptor a 25.2±0.5 fold of basal control increase.

Thus it appears that under the present conditions the VIP_2 and PACAP receptor-mediated [^{3}H]IP responses are not subject to desensitisation whereas the cAMP responses mediated by these receptors are subject to a relatively rapid desensitisation process.

3.3.5 Pertussis toxin-sensitivity of responses mediated by the VIP and PACAP receptors

The effect of PTx pretreatment on VIP and PACAP receptor-mediated responses was assessed. VIP (3 μM) caused an increase in [^{3}H]IP formation to 2.55±0.16 -fold of basal control for the VIP_1 receptor and 1.99±0.05 -fold of basal control for the VIP_2 receptor at a concentration of 3 μM (see Figure 3.8(a)). [^{3}H]IP production mediated by the VIP_1 and VIP_2 receptors was partially inhibited by PTx pretreatment. Treatment of cells with PTx at 100 ng/ml for 16 hours prior to stimulation reduced the VIP-stimulated response by 46±9% for the VIP_1 receptor and 38±10% for the VIP_2 receptor. A PTx treatment of 200 ng/ml had no effect on basal [^{3}H]IP production (100±2% of control). Treatment of cells with the

pharmacologically inactive PTx B subunit (Tamura et al., 1983: at a concentration of 200 ng/ml for 16 hours) had no significant effect on the VIP stimulated [^{3}H]IP production; the VIP_1 receptor maintained 100±3% of its VIP stimulated activity and the VIP_2 receptor 99±7%. A maximal effect of PTx was observed at 100 ng/ml, 200 ng/ml did not cause any greater an inhibition of activity. It might however be advantageous to directly measure PTx activity by incubating membranes with [adenylate-32P]NAD and measuring ADP ribosylation of membrane proteins by SDS-polyacrylamide gel electrophoresis and autoradiography (Burns et al., 1987).

In order to determine whether a pathway activated by the G protein G_s could be responsible for either the PTX-sensitive or -insensitive components of PLC activation the cells were preincubated with cholera toxin (CTx) for 16 hours prior to stimulation (a protocol reported to down-regulate Gs: Eason & Liggett, 1994). A direct measurement of G_s levels by Western blotting could provide a means of determining the effectiveness of the CTx pretreatment. A 16 hour preincubation with CTx at a concentration of 20 µg/ml caused no increase in basal [^{3}H]IP formation (100±3% of the basal value which was 9,348±303 dpm/assay. n=6) and had no effect on VIP-evoked PLC responses. Figure 3.8(b) shows that in cells pretreated with 20 µg/ml CTx for 16 hours the VIP-evoked PLC response mediated by the VIP_1 receptor was 105±6% of control and for the VIP_2 receptor, 95±6%. Treatment of COS 7 cells expressing the VIP_2 receptor with 10 µM forskolin (the adenylate cyclase-stimulating diterpene: Seamon et al., 1981) also caused no increase in [^{3}H]IP production (102±3% of basal control, n=4) indicating that the VIP_2 receptor-mediated PLC stimulation is not dependent on cAMP production (a 20 minute stimulation with 10 µM forskolin increased cAMP levels from 1.11±0.02 to 127.7±16.2 pmol/ml; n=6). It is however possible that simultaneous VIP occupancy of the receptor may alter the response to forskolin.

Figure 3.8(c) also shows the PTx-insensitivity of PLC activity stimulated through the $PACAP_{short}$ and $PACAP_{long}$ receptors. 100 nM PACAP-38 stimulated a 7.71±0.55 -fold of basal control response for the "short" form of the receptor; after treatment with 200 ng/ml PTx this was 7.81±0.20 -fold of basal control. The "long" form of the receptor mediated a 10.59±0.82 -fold of basal control response to 100 nM PACAP-38; after PTx treatment the response was 11.00±0.27 -fold of basal control. PTx treatment alone (200 ng/ml for 16 hours prior to stimulation) had no effect on basal activity (91±9% of basal control).

Agonist-evoked [^{3}H]IP production mediated by the VIP_1 and VIP_2 receptors occurs by a mechanism that is partially inhibited by treatment with pertussis toxin, indicating the involvement of a member of the $G_{i/o/z}$ family of G proteins. This is not true of the PACAP receptor-mediated response which is more likely to occur by the classical $G_{q/11}$-mediated pathway. VIP receptor-mediated [^{3}H]IP production is not dependent on and does not appear to be modulated by cAMP.

PLD activation mediated by the VIP and PACAP receptors

Experiments performed by D.A. McCulloch in this lab demonstrated that both the VIP_1 and VIP_2 receptors showed concentration-dependent activation of PLD (100 nM-1 μM). The 1 μM VIP-elicited response was 1.93±0.13 -fold of basal control for [^{3}H]phosphatidylbutanol production through the VIP_1 receptor and 1.62±0.24 -fold of basal control for the VIP_2 receptor. After PTx pretreatment the responses were 97±4% and 109±6% of the controls respectively for the VIP_1 and VIP_2 receptors (MacKenzie et al., 1998).

GTPγS modulation of ligand binding to the VIP_2 receptor

A functional interaction between the VIP_2 receptor and a PTx-sensitive G protein can also be demonstrated by the effect of PTx-pretreatment on the GTPγS modulation of ligand binding (see Section 2.2.7). The affinity of the receptor for ligand is reduced by increasing the levels of active-state G proteins with increasing concentrations of the non-hydrolysable GTP analogue, GTPγS (DeLean et al., 1980). The GTPγS modulation of [^{125}I]helodermin binding to the VIP_2 receptor was observed for untreated and for PTx-pretreated membranes prepared from COS 7 cells transiently expressing the VIP_2 receptor (see Figure 3.9). The maximum specific binding of [^{125}I]helodermin was 13,344±252 cpm/assay. The resultant concentration effect curve for GTPγS on this specific binding showed a clear reduction in binding which was maximal at approximately 100 nM GTPγS (retaining 70.8±2.2% of the maximum specific binding of [^{125}I]helodermin). PTx-pretreatment reduces the dissociation by 45±6% at 3μM GTPγS. Binding to membranes treated with PTx inactivated by glutaraldehyde-treatment (Burns et al., 1987) was identical to that from untreated membranes at 3μM GTPγS (71.1±1.7% of the maximum specific binding of [^{125}I]helodermin).

This evidence indicates that there was a direct interaction between the VIP_2 receptor and PTx-sensitive G proteins and that these may constitute a significant percentage of the G proteins that associate with this receptor.

In order to confirm that the inhibitory effect of Ptx is not the result of an indirect effect on the VIP_2 receptor, the non-specific G protein activator $AlCl_3$/NaF was used to stimulate [^{3}H]IP production and the effect of Ptx-pretreatment on this stimulation was observed. Treatment of the cells for 30 minutes with 10 μM $AlCl_3$ and 50 mM NaF produced a 6.4±0.4 fold of basal control stimulation of [^{3}H]IP

production (a typical basal value being 8,431±170 dpm/assay). Pretreatment of the cells for 16 hours with 100 ng/ml PTx reduced this stimulation to 5.4±0.2 fold of basal control (81.8±4.3% of the untreated value). This inhibition was statistically significant (Wilcoxon two-sample signed rank test, $P<0.05$). This indicates that Ptx-sensitive G proteins in general are capable of stimulating [^{3}H]IP production in COS 7 cells.

The tetradecapeptide, mastoparan , is a compound which has been reported to activate PTx-sensitive G proteins through binding to the N-terminus of the G protein α subunit (Higashijima et al., 1988; Higashijima & Ross, 1991; Weingarten et al., 1991). This compound was therefore used to assess whether the activation of PTx-sensitive G proteins alone was sufficient to stimulate [^{3}H]IP production in these cells. A clear, though modest, concentration-dependent stimulation of [^{3}H]IP production was observed in these cells on treatment with mastoparan (see Figure 3.10). The maximum response, stimulated by 15 μM mastoparan, was 1.34±0.08 fold of basal control. The mastoparan-stimulated response was inhibited an average of 45.8±10.8% by pretreatment of the cells for 16 hours with 100 ng/ml PTx. This inhibition is statistically significant as assessed by the Wilcoxon matched pairs signed rank test ($p<0.05$). Higashijima et al. (1988) also found that PTx-pretreatment was only partially effective in blocking mastoparan-mediated effects. These data suggest that it is possible that the stimulation of [^{3}H]IP production in COS 7 cells could result from the activation of PTx-sensitive G proteins alone.

3.3.6 The role of Ca^{2+} entry in VIP_2 receptor-mediated [^{3}H]inositol phosphate production

There are increasing numbers of reports of G proteins modulating Ca^{2+} channel activity (Hamilton et al., 1991; Zhu & Ikeda, 1994). These reports most often implicate PTx-sensitive G proteins (Hescheler et al., 1987; Hescheler et al.,

1988; Harris-Warrick, 1988, Ewald et al., 1989; Sweeney & Dolphin, 1992; Kleuss et al., 1991; Dolphin et al., 1991; Dolphin, A.C., 1995). Murthy & Makhlouf (1994) reported the VIP receptor-mediated stimulation of a Ca^{2+} channel through a PTx-sensitive G protein in dispersed gastric smooth muscle cells. In order to determine whether Ca^{2+} entry had a role to play in agonist-evoked VIP_2 receptor-mediated [^{3}H]IP production, the effect of a number of Ca^{2+} channel blockers on this response was assessed.

The calcium ionophore, ionomycin, was used to investigate whether Ca^{2+} influx alone was sufficient to stimulate [^{3}H]IP production in COS 7 cells. A 20 minute application of 10 μM ionomycin stimulated a 7.9±0.5 fold of basal control response (n=4). The basal value was 4,273±343 dpm/assay.

ATP powerfully stimulates PLC activity in COS 7 cells (Kosugi et al., 1992). This response is likely to be mediated by a native purinoreceptor. The $P2Y_1$-$P2Y_4$ and $P2Y_6$ subtypes of metabotropic purinoreceptor have all been shown to couple to PLC stimulation (for review see North & Barnard, 1997). ATP-evoked [^{3}H]IP production was therefore used as a control for the effects of Ca^{2+} channel blockers on VIP_2 receptor-mediated [^{3}H]IP production. A concentration-response curve for ATP-evoked [^{3}H]IP production was performed (see Figure 3.11). The EC_{50} value for ATP-stimulated [^{3}H]IP production was 28.2±0.5 μM and the maximum response 32.9±0.1 fold of basal control. The response stimulated by 50 μM ATP was not PTx-sensitive (16 hours pretreatment with 200 ng/ml PTx). The [^{3}H]IP production levels with or without PTx-pretreatment were 11.7±0.2 and 11.2±0.3 -fold of basal control respectively.

Co^{2+} is a commonly used blocker of divalent cation channels (Hiriart et al., 1988; Rorsman et al., 1986; Plant, 1988). Concentration-response curves for Co^{2+} on both ATP-evoked and PACAP-38-evoked [^{3}H]IP production in COS 7 cells

expressing the VIP_2 receptor were carried out (see Figure 3.12(a)). The VIP_2 receptor-mediated response was clearly inhibited in a concentration-dependent manner by Co^{2+} (to 22±9% of the control response at 0.3 mM Co^{2+}) whereas the ATP-evoked response was unaffected by Co^{2+} treatment (94±4% of the control response at 0.3 mM Co^{2+}).

Methoxyverapamil, the phenylalkylamine blocker of L-type Ca^{2+} channels (dihydropyridine-sensitive (DHP) Ca^{2+} channels), was used by Murthy & Makhlouf (1994) to block the VIP-receptor activated Ca^{2+} channel in gastric smooth muscle cells. Concentration response curves for methoxyverapamil were carried out on both ATP-evoked and PACAP-38-evoked [^{3}H]IP production in COS 7 cells expressing the VIP_2 receptor (see Figure 3.12(b)). No significant inhibition of [^{3}H]IP production mediated by either receptor was observed at concentrations of methoxyverapamil up to 100 µM (101±15 and 82±4% of the control response for the PACAP-38- and ATP-evoked responses respectively). Nifedipine is a 1,4-DHP compound which is a selective and effective blocker of DHP-sensitive Ca^{2+} channels (Nowycky et al., 1985; Fox et al., 1987). Concentrations of up to 10 µM nifedipine had no inhibitory effect on PACAP-38-evoked [^{3}H]IP production mediated by the VIP_2 receptor expressed in COS 7 cells (120±13% of the control response after treatment with 10 µM nifedipine, n=6). SK&F 96365 is a compound described by Merritt et al. (1990) which inhibits both calcium-release activated calcium influx ($CRAC_i$) and DHP-sensitive Ca^{2+} channels. Concentration response curves to investigate SK&F 96365 effects on both ATP-evoked and PACAP-38-evoked [^{3}H]IP production in COS 7 cells expressing the VIP_2 receptor were carried out (see Figure 3.12(c)). This compound was observed to have a marked concentration-dependent inhibitory effect on PACAP-38-evoked [^{3}H]IP production (the response in the presence of 100 µM SK&F 96365 being 16±8% of the control

response). The ATP-evoked response however was only slightly reduced (83±3% of the control response) by treatment of the cells with 100 μM SK&F 96365.

The experiments with Ca^{2+} channel blockers demonstrated that [^{3}H]IP production mediated by the VIP_2 receptor is at least partially dependent on Ca^{2+} channel activity. The ineffectiveness of both methoxyverapamil and nifedipine strongly suggests that the Ca^{2+} channel activity is not attributable to a DHP-sensitive (L-type) Ca^{2+} channel. The inhibitory effect of SK&F 96365 on VIP_2 receptor-mediated [^{3}H]IP production indicates that the Ca^{2+} channel may be of a type activated by Ca^{2+} release from intracellular stores.

In experiments on dorsal root ganglion neurons it was found that GTP analogues potentiated the effect of receptor agonists to inhibit Ca^{2+} currents in a PTx-insensitive manner. However an L-type current remained which was resistant to this inhibition. Scott & Dolphin (1987) therefore examined the effects of the L-type calcium channel antagonists methoxyverapamil (D600), nifedipine and diltiazem on GTPγS modulated Ca^{2+} currents. Surprisingly, all three compounds produced a marked PTx-sensitive potentiation of the Ca^{2+} current in the presence of GTPγS. The effects of nifedipine, methoxyverapamil and SK&F 96365 were examined on [^{3}H]IP production in COS 7 cells stimulated by the non-specific G protein activator $AlCl_3$/NaF (see Table 3.2). Interestingly, a clear and significant potentiation of $AlCl_3$/NaF stimulated [^{3}H]IP production was observed for all three compounds. Treatment with 10 μM nifedipine increased the $AlCl_3$/NaF-stimulated response to 141.9±4.3% of the control response stimulated by 10 μM $AlCl_3$ and 50 mM NaF (10.5±0.5 fold of basal control), 30 μM methoxyverapamil increased the response to 132±5% and SK&F 96365 increased the response to 130±6% of the control response.

All three Ca^{2+} channel blockers had similar effects on $AlCl_3$/NaF stimulated [^{3}H]IP production despite SK&F 96365 being the only one to significantly block PACAP-38-evoked [^{3}H]IP production mediated by the VIP_2 receptor. These data are consistent with the idea that nifedipine, methoxyverapamil and SK&F 96365 can effectively modulate G protein-regulated L-type Ca^{2+} channel activity and thus potentially influence PLC activity in these cells, but this, as outlined above, is not the mechanism by which the VIP_2 receptor mediates PLC activation.

Thapsigargin is a well-established inhibitor of intracellular Ca^{2+} store Ca^{2+} pumps (sarcoplasmic and endoplasmic Ca^{2+}-ATPase(SERCA) inhibitor: Thastrup et al., 1990). By inhibiting refilling of Ca^{2+} stores thapsigargin stimulates calcium-release activated calcium influx ($CRAC_i$). Concentration-response curves were constructed for thapsigargin-evoked [^{3}H]IP production in the presence and absence of PACAP-38 (see Figure 3.13). The cells were treated with thapsigargin and/or PACAP-38. Thapsigargin alone caused a concentration-dependent increase in [^{3}H]IP levels (reaching 30±5% of the control response at a concentration of 3 μM). Thapsigargin caused a significant potentiation of PACAP-38-evoked [^{3}H]IP production at a concentration of 3 μM (an increase of 71±20% over the control response: the responses stimulated by 3 μM thapsigargin, 1 μM PACAP-38 and 1 μM PACAP-38 in the presence of 3 μM thapsgargin were; 1.28±0.03-, 2.75±0.03- and 3.56±0.15-fold of basal control respectively). These data demonstrate that a modest stimulation of [^{3}H]IP production may result from Ca^{2+} influx caused by the emptying of intracellular stores in COS 7 cells and that this mechanism causes a slight potentiation of the VIP_2 receptor-mediated stimulation of [^{3}H]IP production.

The VIP_2 receptor-mediated stimulation of [^{3}H]IP production was partially PTx-sensitive. It is possible that the VIP_2 receptor may stimulate PLC by a number

of different mechanisms, for example, by the interaction of a G protein with a Ca^{2+} channel to stimulate Ca^{2+} influx and by the simultaneous activation of PLC by a G protein. In order to determine whether the Ptx-sensitive component of the VIP_2 receptor-mediated stimulation of [^{3}H]IP production could be associated with the activation of Ca^{2+} channels, the effect of increasing concentrations of Co^{2+} on the PACAP-38-evoked stimulation of [^{3}H]IP production in PTx-treated and untreated cells was examined (see Figure 3.14). In these experiments Ptx-treatment reduced the PACAP-38-evoked response to 70±12% of the control response, while the presence of 0.01 or 0.03 mM Co^{2+} did not reduce this further (values of 70±1 and 74±6% of the control response respectively). Co^{2+} however, caused a clear concentration-dependent reduction in PACAP-38-evoked [^{3}H]IP production in the absence of PTx-treatment. These data indicate that the PTx-sensitive component of the VIP_2 receptor-mediated stimulation of [^{3}H]IP production was more sensitive to Co^{2+} treatment than the PTx-insensitive component suggesting that the activation of PTx-sensitive G proteins by the VIP_2 receptor may well be associated with the stimulation of Ca^{2+} channels.

3.3.7 The involvement of pertussis toxin-sensitive G proteins in agonist-evoked cAMP production mediated by the rat VIP_2 receptor

The stimulation of PLC by PTx-sensitive G proteins is known to occur predominantly as a result of PLC activation by βγ subunits (Lee & Rhee, 1995; Exton, J.H., 1996). There are very few instances of α subunits from PTx-sensitive G proteins being implicated in the agonist-evoked stimulation of PLC (Exton, J.H., 1996). PTX-sensitive G proteins are most commonly associated with the inhibition of AC activity (Taussig et al., 1993; Taussig et al., 1994). It is therefore possible that the PTx-sensitive G proteins activated by the VIP_2 receptor not only stimulate PLC activity, through the action of their βγ subunits, but also cause the inhibition of

adenylate cyclase as a result of their α subunit activity. The effect of PTx-pretreatment on VIP-evoked cAMP production mediated by the VIP_2 receptor transiently expressed in COS 7 cells was therefore examined (see Figure 3.15). The treatment of cells with 200 ng/ml PTX for 16 hours before stimulation with VIP (0.01 nM - 100 nM) had no detectable effect on cAMP production (117.2±3.6 and 117.4±3.9 pmol/ml cAMP at a concentration of 100 nM VIP with and without PTx-treatment respectively). The inhibitory effect of $G_{i/o}$ activation on AC activity depends on the AC isoform involved (Taussig et al., 1993; Taussig et al., 1994). The effect can be quite small and may be difficult to identify over the simultaneous stimulatory effect of G_s. In order to observe the effect of the inhibitory G proteins in such cases it is necessary to remove the influence of the stimulatory G proteins. This can be accomplished by pretreating the cells with 20 μg/ml cholera toxin (CTx) for 16 hours before stimulation with receptor agonist, a protocol known to down-regulate G_s (Eason & Liggett, 1994), and stimulating AC with 10 μM forskolin at the time of adding agonist. This results in a maximally stimulated adenylate cyclase system with reduced G_s levels in which an inhibitory effect on cAMP production should be easily identified. A concentration-response curve for PACAP-38-mediated cAMP production was performed on CTx and forskolin-treated cells (see Figure 3.16). Basal cAMP production was 1.11±0.02 pmol/ml. 10 μM Forskolin applied to CTx-treated cells stimulated the production of 438.±28 pmol/ml cAMP. PACAP-38 at concentrations up to 3 μM caused no apparent inhibition of cAMP production but stimulated an increase in cAMP production (596±43 pmol/ml at 1 μM PACAP-38). It is possible that down regulation of G_s by CTx pre treatment has made this the limiting factor in the VIP_2 receptor-mediated stimulation of adenylate cyclase and has 'right-shifted' the concentration-response curve for PACAP-38-evoked cAMP production to the extent that it falls within the same range as the concentration-response curve for

PACAP-38-evoked [^{3}H]IP production. Alternatively, the VIP_2 receptor may be mediating the stimulation of AC by a mechanism other than G_s activation at high agonist concentrations.

The PTx-sensitive G protein activator mastoparan, at a concentration of 8 μM, caused a clear inhibition of CTx- (20 μg/ml for 16 hours) and forskolin- (10 μM for 10 minutes) stimulated cAMP production in COS 7 cells (from 630±26 to 388±55 pmol/ml; an inhibition of 38±10% of the CTx/forskolin-stimulated response: see Figure 3.17). PTx-pretreatment (100 ng/ml for 16 hours) reversed the inhibition, returning the cAMP levels to 682±29 pmol/ml. PTx-pretreatment had no effect on basal cAMP production but almost doubled the CTx-stimulated level (from 119±9 to 202±24 pmol/ml). CTx and forskolin have a synergistic effect on cAMP production; together producing 630±26 pmol/ml cAMP (forskolin alone stimulated the production of 199±21 pmol/ml cAMP). Ptx-treatment increased the CTx/forskolin stimulated level of cAMP production from 630±26 to 680±24 pmol/ml. This slight increase is not significant but is probably equivalent to the increase observed after PTx-treatment of CTx-treated cells. These data demonstrate that the activation of adenylate cyclases in COS 7 cells by CTx and forskolin is subject to inhibition by treatment with mastoparan, the presumed $G_{i/o/z}$ activator. This inhibition is completely reversed by PTx-treatment suggesting that it is mediated by PTX-sensitive G proteins. In conclusion, under the present conditions, an inhibition of AC by Ptx-sensitive G proteins activated by the VIP_2 receptor could not be demonstrated.

3.3.8 The effect of various kinase inhibitors on [^{3}H]inositol phosphate production mediated by the VIP_2 and related receptors transiently expressed in COS 7 cells

In order to determine whether agonist-evoked VIP_2 receptor-mediated stimulation of [^{3}H]IP production in COS 7 cells was modulated by protein kinases a number of protein kinase inhibitors were tested on this response. Certain of the inhibitors were also tested on related receptors (see Table 3.3).

Treatment of cells with the PKC inhibitor GF 109203X (Toullec et al., 1991), at a concentration of 10 μM, caused the VIP_1 and VIP_2 receptor-mediated VIP-evoked responses to increase slightly but not in a statistically significant manner (117±9 and 122±6% of their control responses, which were; 2.84±0.19 and 2.03±0.04 fold of basal control respectively). The VIP_1 and VIP_2 receptor-mediated VIP-evoked responses were also unaffected by treatment of the cells with 30 μM H-89, an isoquinolinesulfonamide inhibitor of cAMP-dependent protein kinase (PKA) (Chijawa et al., 1990). The values after treatment were 100±8 and 108±16% of the control responses for the VIP_1 and VIP_2 receptors respectively. The treatment of cells with the non-specific protein kinase inhibitor, staurosporine (Tamaoki et al., 1986), at a concentration of 1 μM, increased VIP_1, VIP_2 and $PACAP_{long}$ receptor-mediated agonist-evoked [^{3}H]IP production to 175±51, 257±42 and 173±19% of their respective control responses (7.74±0.63 fold of basal control was the control response for the $PACAP_{long}$ receptor-mediated PACAP-38 -evoked activity). The $AlCl_3$/NaF stimulated response was similarly increased to 162±1% of the control response, which was 13.4±0.3 fold of basal control. This indicates that staurosporine is likely to be active at a point downstream of the receptor e.g. at a G protein or PLC itself. Treatment of cells with the tyrosine kinase inhibitor, genistein

(Akiyama & Ogawara, 1991), at a concentration of 100 μM had no effect on VIP_2 receptor or $PACAP_{long}$ receptor-mediated [^{3}H]IP production (83±11 and 103±4% of their control responses respectively after treatment).

These data indicate that there are no apparent tonic effects of GF 109203X-sensitive PKC isoforms, H 89-sensitive PKA isoforms or genistein-sensitive tyrosine kinases on VIP_2 receptor-mediated stimulation of [^{3}H]IP production in COS 7 cells. The $PACAP_{long}$ receptor-mediated stimulation of [^{3}H]IP production is similarly unaffected by genistein-sensitive tyrosine kinases and the VIP_1 receptor-mediated stimulation of [^{3}H]IP production is unaffected by GF 109203X-sensitive PKC isoforms and H 89-sensitive PKA isoforms. The agonist-evoked [^{3}H]IP production mediated by all three receptors was potentiated by staurosporine treatment of the cells but this was also true of the response stimulated by the non-specific G protein activator $AlCl_3$/NaF indicating that the effect of staurosporine or its target kinase(s) is downstream of the receptor at a G protein or possibly PLC itself.

3.3.10 Preliminary experiments on immunoprecipitation and immunoblotting for VIP_2 receptor-associated G proteins

In order to identify the G proteins associated with the VIP_2 receptor which were involved in PLC stimulation, a human VIP_2 receptor construct with a haemagluttinin(HA) tag sequence attached to its C-terminus was used. The human VIP_2 receptor sequence varies from the rat receptor sequence in a limited number of residues (see Table 4..3). As a means of confirming that there were no major differences in the signalling characteristics of the tagged human and the rat isoforms of the receptor, the HA-tagged human receptor was expressed in COS 7 cells and a concentration-response curve for PACAP-38-evoked [^{3}H]IP production was carried out (see Figure 3.18). The human receptor mediated a 1.73±0.10 fold of basal control response with an EC_{50} value of 66.7±9.7 nM. The stimulation of [^{3}H]IP

production was PTx-sensitive, being inhibited to 65±11% of the 1 μM PACAP-38-evoked response (1.58±0.12 fold of basal control) by 16 hours pretreatment with 100 ng/ml PTx. The HA-tagged human VIP_2 receptor, when expressed in HEK293 cells, behaves identically to the untagged human VIP_2 receptor with respect to VIP-evoked cAMP production (EC_{50} values of 1.4±0.2 and 1.2±0.2 nM respectively: Diane Dinnis; personal communication). The HA-tagged human VIP_2 receptor-mediated signalling appears to be very similar to that of the rat VIP_2 receptor. It is therefore likely that the two receptors will interact with equivalent G proteins when expressed in the same cell line.

The HA-tagged human VIP_2 receptor was expressed in COS 7 cells and the receptor was immunoprecipitated from a solubilised membrane preparation of the cells using an antibody specific for the HA tag (see section 2.2.13). The conditions for receptor solubilisation and specific immunoprecipitation under conditions retaining associated proteins have been extensively characterised by other workers in the lab. Western blots of the immunoprecipitate were immunostained with antibodies for $G\alpha_q$, $G\alpha_{i1/2}$ and $G\alpha_{i/o/t/z}$. To confirm the specificity of staining, antisera were preincubated (15 minutes, 20°C) with a specific peptide antigen.

In these experiments the presence of $G\alpha_q$ was clearly detected in the immunoprecipitate (see Figure 3.19(c)) as was a member of the $G\alpha_{i/o/t/z}$ family (see Figure 3.19(b)). The specific bands identified by their anti-sera were approximately 42 and 47 kDa in size respectively and were blocked by preincubation with the appropriate peptide antigen. The antiserum for $G\alpha_{i1-2}$ produced no bands which were convincingly blocked by the appropriate peptide antigen (see Figure 3.19(a)). The data therefore provide evidence to suggest that a PTx-sensitive G protein (other than $G_{i1/2}$) and G_q may associate closely with the VIP_2 receptor.

3.4 Discussion

Consistent with present findings there is considerable evidence in the literature that the PACAP receptor mediates AC and PLC activation and that VIP_1 and VIP_2 receptors mediate AC activation. When expressed in COS 7 cells the PACAP receptor was represented at a higher level than the VIP_2 receptor and mediated a higher fold stimulation of cAMP production (see Table 3.1). It has been known for some time that the PACAP and VIP_2 receptors stimulate AC in COS 7 cells (Morrow et al., 1993; Lutz et al., 993) and that agonist activation of the PACAP receptor additionally stimulates PLC and mobilises intracellular Ca^{2+} (Gottschall et al., 1991; Deutsch & Sun., 1992; Tatsuno et al., 1992). Our observations provide evidence that VIP_1 and VIP_2 receptors are also capable of activating PLC.

3.4.1 Agonist-evoked [^{3}H]inositol phosphate production mediated by the rat VIP_2 receptor

There are a number of reports in the literature suggesting that VIP-preferring receptors have the ability to activate PLC. Many reports describe the effects of VIP at concentrations which are consistent with VIP_1 and VIP_2 receptor-mediated effects. However, few of the early studies carried out pharmacological characterisation such as the relative potencies of VIP and PACAP-38 which could lead specifically to identification of VIP receptor-mediated rather than PACAP receptor-mediated effects. VIP concentrations in the micromolar range have been shown to produce an increased level of phosphatidyl inositol (PI) turnover in superior cervical ganglion (Audigier et al., 1986). Malhotra et al. (1988) found VIP and muscarine to stimulate PI hydrolysis and mobilise intracellular Ca^{2+} stores

inducing the secretion of catecholamines in adrenal chromaffin cells. Low concentrations of VIP were also sufficient to activate PKC in regions of rat hippocampus (Weill et al., 1989). Low levels of VIP were shown to mobilise calcium in astroglial cells derived from rat cerebral cortex (Russell et al., 1990) and Fatatis et al. (1994) observed that in type 1 astrocytes from rat cerebral cortex, α-adrenoreceptors were found to act synergistically with VIP receptors to augment an intracellular Ca^{2+} signal which was abolished by thapsigargin treatment. The PI hydrolysis mediated by the VIP and α-adrenoreceptors did not appear to be additive (Fatatis et al., 1994). Inagaki et al. (1994) cloned a VIP_2 receptor from the mouse insulin-secreting cell line MIN6. Expression of this receptor in *Xenopus* oocytes showed that calcium-activated chloride currents were stimulated by PACAP suggesting that the emptying of intracellular Ca^{2+} stores as a result of PLC stimulation had taken place.

These examples strongly suggest that VIP receptors can stimulate PI hydrolysis. When this study began, no direct evidence of such a mechanism had been presented. In this study the ability of the VIP_2 receptor to stimulate the production of [^{3}H]IP has been clearly demonstrated in COS 7 cells (see Figure 3.3, Figure 3.4 and Table 3.1). VIP and PACAP-38 have been shown to be equipotent for [^{3}H]IP production mediated by the VIP_2 receptor, as they were for cAMP production mediated by this receptor. The scale of the [^{3}H]IP production mediated by the VIP_2 receptor is smaller than that mediated by the PACAP receptors (see Figure 3.4 & Table 3.1) and the potency of PACAP-38 is less potent at the VIP_2 receptor. This may be attributable to the higher expression level of the PACAP receptors, a different affinity of interaction with the same G proteins or a different mechanism being in operation.

It has since been shown that the human VIP_1 receptor stably expressed in HEK 293 and CHO cells is capable of mediating VIP-evoked increases in intracellular Ca^{2+} (Sreedharan et al., 1994). Transient and sustained phases of Ca^{2+} increase were observed in response to VIP. In stably transfected HEK293 cells, the transient phase of Ca^{2+} increase was abolished by thapsigargin treatment, indicating that it was due to the release of Ca^{2+} from intracellular stores, and the sustained phase was abolished by removing extracellular Ca^{2+}, indicating that it was probably due to Ca^{2+} entry through channels in the plasma membrane (Sreedharan et al., 1995). PI hydrolysis and the cAMP response were unaffected by this treatment. The stimulation of PI hydrolysis and Ca^{2+} responses were inhibited by cholera toxin or forskolin treatment indicating that cAMP modulated the IP response. This is not true of the IP response mediated by the VIP_1 and VIP_2 receptors expressed in COS 7 cells. A human intestinal epithelium cell line (HT29 cells) expresses a low level of VIP_1 receptors (Sreedharan et al., 1993) and VIP has also been shown to stimulate transient Ca^{2+} increases in these cells (Sreedharan et al., 1994). Van Rampelbergh et al. (1997) have recently shown that the rat PACAP and VIP_1 receptors, when stably expressed in CHO cells, can mediate the stimulation of both adenylate cyclase and PLC and that the VIP_1 receptor-mediated stimulation of PLC is PTx-sensitive. These authors found that a comparison of clones expressing similar amounts of the PACAP and VIP_1 receptor revealed that the PACAP receptor still mediated a higher fold stimulation of PLC. Although the EC_{50} values are referred to in the text of that publication, the authors chose not to quote the actual values simply describing them as being in the nanomolar range. The illustrations in that publication indicate that agonist-evoked stimulation of PLC became maximal at between 3-5 nM for the PACAP receptor-mediated response and at approximately 100 nM for the VIP_1 receptor-mediated response.

3.4.2 The pertussis toxin sensitivity of [^{3}H]inositol phosphate production mediated by the rat VIP_2 receptor

Murthy et al. (1993) described the stimulation of NOS mediated by a VIP receptor in dispersed gastric smooth muscle cells. This is presumed to contribute to smooth muscle relaxation (a topic discussed in Chapters 1 and 6). The concomitant activation of PKC was found to inhibit NOS activity (Murthy et al., 1994) the stimulation of which was postulated to occur by a direct G protein interaction with NOS and an indirect Ca^{2+} influx mechanism, one or both of which are attributable to the activation of a PTx-sensitive G protein. The VIP and PACAP-27-mediated stimulation of NOS was inhibited by antibodies to $G\alpha_{i1/2}$. The VIP receptor in rat lung however, has been demonstrated by cross-linking studies to be capable of coupling to both G_s (Kermode et al., 1992) and $G\alpha_{i3}$ (Diehl & Shreeve, 1995).

An investigation of the PTx-sensitivity of VIP receptor-mediated stimulation of [^{3}H]IP production revealed a significant but partial inhibition of both the VIP_1 and VIP_2-receptor mediated responses after PTx-treatment of the cells (approximately 40% inhibition at 100 ng/ml PTx: see Figure 3.8a). PTx-treatment was also demonstrated to attenuate the GTPγS -mediated reduction in ligand binding to the VIP_2 receptor (approximately 45%: see Figure 3.9). This strongly suggests that the VIP_2 receptor interacts directly with a PTx-sensitive G protein and that this PTx-sensitive G protein constitutes a large percentage of the G proteins which associate with the receptor. The effect of PTx on VIP receptor-mediated IP responses was not attributable to the removal of an inhibitory influence on cAMP production since CTx pre-treatment had no significant effect on [^{3}H]IP production mediated by the VIP receptors (see Figure 3.8(b)) and PTx-treatment had no effect on VIP-evoked cAMP production mediated by the VIP_2 receptor (see Figure 3.15). The PACAP receptors when transiently expressed in COS 7 cells mediated an

agonist-evoked stimulation of [^{3}H]IP production which was not PTx-sensitive (see Figure 3.8(c)) as did the presumed purinoreceptor in COS 7 cells (see Figure 3.11). The PTx-insensitivity of the PACAP receptor-mediated response demonstrates that the high expression level of a non-native receptor in this cell line is not sufficient to cause the activation of PLC by PTx-sensitive G proteins and that the characteristics of the receptor itself are the dominant factors.

The stimulation of [^{3}H]IP production in a PTx-sensitive manner by $AlCl_3$/NaF suggests that the activation of PTx-sensitive G proteins is sufficient to stimulate [^{3}H]IP production in COS 7 cells (stimulation was inhibited approximately 18% by PTx-treatment). The ability of mastoparan to stimulate [^{3}H]IP production in a PTx-sensitive manner (see Figure 3.10) confirms a role for PTx-sensitive G proteins in this system.

Immunoprecipitation of the haemagluttinin-tagged human VIP_2 receptor expressed in COS 7 cells and immunoblotting for the associated G proteins revealed preliminary evidence for the direct association of a member of the $G_{i/o/t/z}$ family of PTx-sensitive G proteins (other than $G_{i1/2)}$ and G_q with the receptor. The PTx-insensitive component of the VIP_2 receptor-mediated stimulation of PLC may therefore be explained by the coupling of G_q to this receptor, in addition to the PTx-sensitive G protein.

βγ subunit stimulation of PLC

There have been many cases now in which the stimulation of PLC by PTx-sensitive G proteins has been reported. There have been very few cases in which an isoform of PLCβ has been shown to be responsive to the α subunits of PTx-sensitive G proteins, βγ subunits however, have been shown to cause stimulation independently of the α subunits (Smrcka & Sternweis, 1993; Boyer et al., 1992;

Park et al., 1993). The potency of βγ stimulation of purified PLCβ isoforms has been determined as being: PLC $\beta_3 > \beta_2 > \beta_1$ (Smrcka & Sternweis, 1993; Park et al., 1993), PLCβ4 is insensitive to βγ stimulation (Jiang et al., 1994). The order of potency of G_q stimulation of PLCβ isoforms is: PLC $\beta_1 \geq \beta_3 > \beta_4 > \beta_2$ (Lee & Rhee, 1995). From this it appears that the β1 isoform appears to be primarily $G\alpha_q$ stimulated whereas PLCβ_2 is primarily βγ–stimulated. The PLCβ_3 isoform however, appears to be responsive to both α and βγ subunits. This indicates that the selective expression of PLC isoforms may be a mechanism for controlling PIP_2 hydrolysis in the cell. Most cells contain members of the $G_{q/11}$ and $G_{i/o}$ families of G proteins; members of the latter family constituting 1-2% of the protein in bovine brain (Sternweis & Robishaw, 1984). The concentration of βγ subunits required to stimulate PLC is generally found to be two orders of magnitude greater than for $G\alpha_q$ (Exton, 1997), which may explain why the EC_{50}s for VIP_2 receptor-mediated [^{3}H]IP production in COS 7 cells are so much greater than those for PACAP receptor-mediated stimulation.

Distribution of PLC isoforms activated by G proteins/Ca^{2+}

The β1 and β3 isoforms of PLC are widespread in cells, β1 is widespread in the brain expressed mainly in the cerebral cortex and hippocampus, β3 is expressed at low level throughout the brain (Rhee et al., 1991; Jhon et al., 1993). The β2 isoform appears to be restricted to haematopoetic cells (Park et al., 1992) and the β4 isoform is associated with the retina (Lee et al., 1993) although it is expressed in the cerebellum and at low levels in other areas of the brain (Kim et al., 1997). The PLCδ isozyme was believed to be regulated by Ca^{2+} alone, but there are now reports of its being stimulated by a poorly understood high molecular weight GTP-binding protein called G_h (Feng et al., 1996) and a Rho-GAP-like protein (Homma & Emori, 1995).

3.4.3 The role of Ca^{2+} influx in VIP_2 receptor-mediated stimulation of [^{3}H]inositol phosphate production

There are now many examples of G protein modulation of ion-channel activity, especially involving the intensively studied L-type Ca^{2+} channels. The majority of these cases involve the activity of PTx-sensitive G proteins (for reviews see: Schultz et al., 1990; Hille, 1994; Wickman & Clapham, 1995; Fasolato et al., 1997).

GH_3 cell membranes contain large amounts of G_o and at least two isoforms of G_i (Rosenthal et al., 1988). It was found that PTx pre-treatment abolished both stimulatory and inhibitory effects on calcium currents in GH_3 cells . Offermans et al. (1989) confirmed this result by demonstrating that the GTPase activity stimulated by both stimulatory and inhibitory hormones in GH_3 cells is PTx-sensitive. Ca^{2+} currents in cardiac and skeletal myocytes are stimulated by cAMP-dependent phosphorylation (Trautwein & Hescheler, 1990) where the G protein-regulated channels are apparently L-type channels (Imoto et al., 1988; Mattera et al., 1989; Yatani et al., 1987). In adrenocortical and pituitary cells however this is not the case; intracellularly applied cAMP or extracellular forskolin do not stimulate Ca^{2+} currents. In an adrenocortical cell line, Y1 cells, which contain no G_o, angiotensin II stimulated a voltage-dependent Ca^{2+} current(VDCC) which involved a PTx-sensitive G protein but was independent of cAMP production (Hescheler et al., 1988).

In work carried out on rat cerebral cortical membranes Sweeney & Dolphin (1992) found that L-type 1,4-dihydropyridine sensitive Ca^{2+} channels act as GTPase-activating proteins(GAP) to stimulate GTP hydrolysis by $G\alpha_o$.

Dihydropyridine agonists were found to stimulate the GTPase activity. Because this was a membrane-delimited system, any effect on soluble second messengers was excluded. A PTx-sensitive G protein was also involved in α_{2A}-adrenoreceptor-mediated stimulation of Ca^{2+} influx in rat portal vein smooth muscle cells (Lepretre & Mironneau, 1994). The Ca^{2+} entry was blocked by oxidipine (a VDCC blocker) and whole-cell patch clamp experiments demonstrated that the Ca^{2+} influx occurred through L-type Ca^{2+} channels. The activation of Ca^{2+} influx by both the receptor and mastoparan was reduced by PTx-treatment and a PKC inhibitor (GF 109203X). This was consistent with the hypothesis that a PTx-sensitive G protein was involved in a pathway leading to the activation of PKC ,which then phosphorylates the channel, thereby increasing its activity.

As was mentioned in section 3.3.6, the VIP receptor in rabbit gastric smooth muscle cells has been implicated in the direct stimulation of a Ca^{2+} channel by a PTx-sensitive G protein ($G_{i1/2}$: Murthy et al. 1993; Murthy & Makhlouf, 1994). Initially the authors found that treatment of the cells with 1 μM methoxyverapamil (a phenylalkylamine, L-type Ca^{2+} channel blocker) completely inhibited VIP-evoked NO production and cGMP production. In the later publication the authors provide data indicating that, in addition to VIP-evoked Ca^{2+} influx, a direct stimulation of NOS by a G protein may also occur. In that system PKC activation effectively blocked the Ca^{2+}-mediated responses indicating that VIP receptor stimulation did not result in PKC activation in rabbit gastric smooth muscle cells.

Ionomycin was shown to very effectively activate [^{3}H]IP production in COS 7 cells demonstrating that Ca^{2+} influx alone was sufficient to activate PLC in this system (see section 3.3.6). Treatment of the cells with the divalent cation channel blocker Co^{2+} effectively blocked the VIP_2 receptor-mediated stimulation of [^{3}H]IP production, whilst having little effect on the ATP-evoked response in COS 7 cells

(see Figure 3.12(a)). This strongly suggests that there is a role for Ca^{2+} influx in the VIP_2 receptor-mediated response. The rat VIP_2 receptor when expressed in COS 7 cells mediates a stimulation of [^{3}H]IP production which is unaffected by the L-type Ca^{2+} channel blockers, methoxyverapamil, at concentrations up to 100 μM, or nifedipine, at concentrations of up to 10 μM (see Figure 3.12b). These compounds are the most commonly used blockers of L-type Ca^{2+} channels and their ineffectiveness strongly suggests that the VIP_2 receptor does not stimulate Ca^{2+} influx through this class of channel.

Calcium-release activated calcium influx

In human neutrophils Wenzelseifert et al. (1996) found that thapsigargin-stimulated cation entry involved a PTx-sensitive G protein and a non-selective cation channel which was sensitive to SK&F 96365 and Gd^{3+}. The protein phosphatase(PP)1/2A inhibitors, calyculin A and okadaic acid, reduced cation entry whereas inhibition of PP2B with cyclosporin A and FK-506 potentiated the influx. Thapsigargin-stimulated Ca^{2+} influx was partially inhibited by PTx pre-treatment. Thapsigargin also behaved as a partial secretagogue in human neutrophils, activating lysozyme release. This activity was inhibited by the removal of extracellular Ca^{2+}, PTx pre-treatment or SK&F 96365 treatment which strongly suggests that the secretagogue activity was dependent on Ca^{2+} influx mediated by a PTx-sensitive G protein. VIP acts as a secretagogue in a number of tissue and cell types, for example, for prolactin in GH_3 cells (Rostene, 1984), IL-6 in anterior pituitary cells (Tatsuno et al., 1991; Matsumoto et al., 1993; Spangelo et al., 1990; Spangelo et al., 1991) and ACTH secretion in AtT-20D16v cultures (Braas et al., 1994). Further evidence in support of the description of a PTx-sensitive mechanism of Ca^{2+}-release activated Ca^{2+} influx($CRAC_i$) by Wenzelseifert et al. (1996) was provided by Berven et al. (1995), who identified G_{i2} as being required

for $CRAC_i$ activation in hepatocytes. It was found to act between the release of calcium from intracellular stores (stimulated by vasopressin or thapsigargin) and the Ca^{2+} channel in the plasma membrane. An anti-$G_{i1/2}$ antibody and a synthetic $G\alpha_{i2}$ C-terminal peptide inhibited vasopressin- and thapsigargin-stimulated Ca^{2+} entry without affecting vasopressin-induced release from Ca^{2+} stores and an anti-G_q antibody inhibited vasopressin-stimulated Ca^{2+} entry and partially inhibited release from intracellular stores without affecting thapsigargin-stimulated release. In these hepatocytes immunofluorescence studies revealed that G_{i2} was distributed through the cell interior as well as being at the plasma membrane whereas $G_{q/11}$ was found principally at the plasma membrane. This demonstrated that only vasopressin-evoked Ca^{2+} release from intracellular stores is dependent on G_q, whereas both vasopressin- and thapsigargin-evoked Ca^{2-} influx is dependent on a mechanism involving G_{i2}. It would therefore appear that there may be a requirement for a PTx-sensitive G protein in calcium-release activated calcium influx in a number of cell types.

SK&F 96365 is structurally distinct from the known calcium channel antagonists. This compound was first described by Merritt et al. (1990) and was found to block $CRAC_i$ in both non-excitable and excitable cells The IC_{50} for inhibition of $CRAC_i$ by SK&F 96365 in platelets was 8.5 μM for ADP- and 11.7 μM for thrombin-stimulated influx whereas SK&F 96365 had no effect on Ca^{2+} release from internal stores. Similar effects were observed in neutrophil and platelet preparations. Voltage-gated Ca^{2+} entry in GH_3 and rabbit ear-artery smooth muscle cells was inhibited by SK&F 96365 (with IC_{50} values of 3.6 μM and 20-30 μM respectively) whereas the activity of the P2X purinoreceptor in rabbit ear-artery smooth muscle cells was unaffected. SK&F 96365 does not therefore appear to discriminate between voltage-gated- and receptor-mediated- Ca^{2+} entry, inhibiting both within the same concentration range. However, based on the P2X

purinoreceptor results, SK&F 96365 appears to discriminate between different types of receptor-mediated calcium influx. This is a very hydrophobic compound but its presence in the membrane does not disrupt general channel function as demonstrated by its lack of effect on the ionotropic purinoreceptor activity.

The VIP_2 receptor-mediated stimulation of [^{3}H]IP production was inhibited by treatment with SK&F 96365 (see Figure 3.12(c)). The IC_{50} was approximately 30 μM which is similar to the values quoted by Merritt et al. (1990) for inhibition of Ca^{2+} entry. In conjunction with the inability of the L-type Ca^{2+}-channel blockers (methoxyverapamil and nifedipine) to inhibit the VIP_2 receptor-mediated stimulation of [^{3}H]IP production the data suggest that a calcium channel (other than an L-type channel) activated by the emptying of intracellular stores is involved. Thapsigargin was observed in this system to stimulate [^{3}H]IP production and to potentiate VIP_2 receptor-mediated stimulation of [^{3}H]IP production (see Figure 3.13). This constitutes further evidence that $CRAC_i$ is a relevant mechanism for VIP_2-receptor mediated stimulation of [^{3}H]IP production. The precise nature of the channel is unknown. It may be one of the voltage-dependent calcium channels, which are highly selective for calcium, or a calcium-permeable cation channel, these channels show little or no selectivity for Ca^{2+}, are insensitive to organic calcium-channel ligands and may also lack a voltage-gating mechanism (Schultz et al., 1990).

As mentioned above, the VIP receptor in rabbit gastric smooth muscle cells is linked to NOS activation (Murthy et al., 1993), a topic which will be explored further in Chapter 5. In rat thoracic aorta the Ca^{2+}-ATPase inhibitors thapsigargin and cyclopiazonic acid(CPA) induced NO-mediated cGMP production and relaxation. This was blocked by SK&F 96365 but not nifedipine. The nitroprusside or A23187-induced relaxation was unaffected by SK&F 96365. ACh-induced

relaxation and cGMP production were blocked by SK&F 96365. The data indicates that endothelium-dependent relaxation of vascular smooth muscle is due to the activation of a constitutive NOS by calcium-release activated Ca^{2+} influx. CPA- and thapsigargin-induced endothelium-dependent relaxation and cGMP production in rat thoracic aorta were inhibited by NOS inhibitors, calmodulin inhibitors and removal of Ca^{2+}, suggesting that NO is involved in the relaxation induced by Ca^{2+}-ATPase inhibitors (Moritoki et al., 1994;1996; Zheng et al., 1994). The conclusion is therefore that Ca^{2+}-ATPase inhibitors deplete intracellular stores promoting Ca^{2+} influx which activates cNOS and causes NO production which in turn activates guanylate cyclase causing cGMP production and relaxation of vascular smooth muscle (Kondoh et al., 1993; Moritoki et al., 1994a,b).

Ca^{2+} is recognised as playing a central role in NO production in the vascular endothelium (Mulsch et al., 1989; Schmidt et al., 1989). In endothelial cells agonist-induced increases in Ca^{2+} concentration have been attributed both to the release of Ca^{2+} from intracellular stores and influx via Ca^{2+} channels (Schilling et al.,1988; 1989; 1992; Jacob, 1990; Dolor et al., 1992). Ku & Williams (1995) also observed that thapsigargin selectively increases endothelial Ca^{2+}-concentration and produces both prostanoid and nitric oxide-dependent relaxation in canine coronary arteries.

The VIP_2 receptor has been shown through preliminary studies using immunoprecipitation and Western blotting to be associated with both PTx-sensitive G proteins and G_q (see Figure 3.20). Since the VIP_2 receptor-mediated stimulation of [^{3}H]IP production is only partially PTx-sensitive it is possible that these two G proteins are involved in different aspects of this stimulation, for example the PTx-sensitive G protein may be involved in the Ca^{2+} channel activity while G_q directly activates PLC. The co-immunoprecipitation of these G proteins with the VIP_2 receptor is of course not evidence of a functional interaction. The PTx-sensitive

phase of the component of the VIP_2 receptor-mediated [^{3}H]IP production however appears to be more sensitive to the effects of Co^{2+} than the PTx-resistant component (see Figure 3.14). This is fully consistent with the idea that a PTx-sensitive G protein is involved in the activation of the Ca^{2+} channel. Additional experiments such as the use of a G_q antiserum in a membrane PLC assay would be required in order to positively identify a role for G_q in this system. Although a portion of the response appears to be PTx-insensitive this could also be due to the incomplete ADP-ribosylation of $G_{i/o}$ in this system.

Scott & Dolphin (1987) made the interesting observation that L-type Ca^{2+} channel antagonists (nifedipine, diltiazem and SK&F 96365) actually potentiated Ca^{2+} currents through L-type channels in dorsal root ganglion neurons on the application of GTPγS. This was attributed to activated G proteins stabilising the channel in a state in which the high affinity binding site was converted from antagonist to agonist form such that antagonists developed agonist activity. Sanguinetti et al. (1986) reported that DHP agonists bind preferentially to the resting state of the channel, which is consistent with the idea that activation of the L-type channel causes a change in the characteristics of its binding sites. Nifedipine, methoxyverapamil and SK&F 96365 had a similar potentiating effect on $AlCl_3$/NaF-stimulated [^{3}H]IP production in COS 7 cells to that described by Scott & Dolphin (1987) on GTPγS modulated Ca^{2+} currents through L-type channels in dorsal root ganglion neurons (see Table 3.2). Although of course, methoxyverapamil and nifedipine, had little effect on VIP_2 receptor-mediated [^{3}H]IP production, whereas SK&F 96365 was inhibitory (see Figure 3.12). The probable reason for the different effects of the inhibitors on VIP_2 receptor-mediated and $AlCl_3$/NaF stimulated [^{3}H]IP production is that the receptor-mediated response does not involve the stimulation of L-type Ca^{2+} channels and therefore is unaffected by compounds such as nifedipine and methoxyverapamil which are believed to be

active only at L-type Ca^{2+} channels. It is therefore likely that the broad spectrum G protein activator, $AlCl_3$/NaF, activates G proteins which stimulate Ca^{2+} influx through L-type Ca^{2+} channels, in addition to the channels activated by VIP_2 receptor-mediated signalling, and that the VIP_2 receptor-activated channels are likely to be a minority in the Ca^{2+} channel population activated by $AlCl_3$/NaF. The inhibitory effect of SK&F 96365 on the subtype of Ca^{2+} channel activated by the VIP_2 receptor would therefore be obscured by the potentiating effect on the more abundant L-type Ca^{2+} channels.

In this study it has been demonstrated, by ionomycin treatment, that Ca^{2+} influx alone is sufficient to stimulate [^{3}H]IP production (see Section 3.3.6) and, through Co^{2+} inhibition of PACAP-38-evoked [^{3}H]IP production (see Figure 3.12(a)), that the activity of a divalent cation channel is a central part of the VIP_2 receptor-mediated mechanism. It has been shown that L-type Ca^{2+} channel blockers, despite being active on $AlCl_3$/NaF-evoked [^{3}H]IP production (see Table 3.2), have no effect on VIP_2 receptor mediated [^{3}H]IP production but that SK&F 96365, an inhibitor of L-type Ca^{2+} channels and $CRAC_i$, is an effective inhibitor of this activity (see Figure 3.12). It has also been shown, through CTx treatment, that cAMP does not modulate [^{3}H]IP production (see Section 3.3.5), providing further evidence against a role for L-type Ca^{2+} channels (which are known to be activated by PKA phosphorylation (Tsien et al., 1987; Armstrong & Eckert, 1987)) in VIP_2 receptor mediated [^{3}H]IP production. Thapsigargin treatment has been shown to both stimulate [^{3}H]IP production and potentiate VIP_2 receptor-mediated [^{3}H]IP production (see Figure 3.13), demonstrating that the emptying of intracellular Ca^{2+} stores is part of a mechanism whereby PLC can be activated in COS 7 cells and may be part of the VIP_2 receptor-mediated mechanism. Wenzelseifert et al. (1996) and Berven et al. (1995) described a role for PTx-sensitive G proteins in $CRAC_i$ activation. In this study, the Co^{2+} sensitivity of the PTx-sensitive component of

VIP_2 receptor-mediated [^{3}H]IP production (see Figure 3.14) also suggests that a PTx-sensitive G protein is involved in the mechanism of VIP_2 receptor-mediated Ca^{2+} influx.

A number of additional experiments must be carried out in order to accumulate sufficient data to allow a detailed model of the system to be formulated. An assay of VIP_2 receptor-mediated PI hydrolysis in a membrane-delimited system (i.e. performed on membranes prepared from COS 7 cells transiently expressing the VIP_2 receptor) would answer several pertinent questions. Agonist-evoked PI hydrolysis under these conditions would demonstrate that the response is not entirely attributable to Ca^{2+} channel activity. Treatment of the membranes with antiserum specific to $G\alpha_q$ and $G\alpha_{i/o/z}$ would allow an assessment of which of these G proteins is likely to act between the receptor and PLC, and perhaps even a quantification of the relative extent to which each contributed to the response, if any. It would then be instructive to examine the Ca^{2+} responses involved in the VIP_2 receptor-mediated activation of PLC. Fura-2 fluorescence dye spectroscopy may be the simplest way to accomplish this. This method would allow us to determine whether the VIP_2 receptor-mediated response involves the transient Ca^{2+} spike followed by the sustained increase in intracellular Ca^{2+}, associated with emptying of intracellular stores and influx of Ca^{2+} through a plasma membrane channel, respectively. The PTx-sensitivity of each of those responses could be determined and then the PTx-sensitivity of thapsigargin-stimulated Ca^{2+} influx in order to identify the phase(s) of the response dependent on the PTx-sensitive G protein(s). The role of PLC in the VIP_2 receptor-mediated increase in intracellular Ca^{2+} could be determined by using the PLC inhibitor U73122. This would allow the identification of a non-PLC mediated increase in intracellular Ca^{2+} e.g. the direct activation of a Ca^{2+} channel by a PTx-sensitive G protein. Further experiments involving various Ca^{2+} channel inhibitors would be

useful, although appropriate characterisation of any Ca^{2+} channels involved would be best carried out using electrophysiological techniques such as patch-clamping in combination with specific inhibitors of different Ca^{2+} channel types.

3.4.4. The role of PTx-sensitive G proteins in VIP_2 receptor-mediated cAMP production

Since VIP_2 receptor mediated stimulation of [^{3}H]IP production was found to involve a PTx-sensitive mechanism, an investigation of the role of PTx-sensitive G proteins in VIP_2 receptor-mediated cAMP production was carried out. However it was not possible to identify any effect of PTx-pre-treatment on VIP_2 receptor mediated cAMP production (see Figure 3.15) indicating that there was no tonic effect of a PTx-sensitive G protein on VIP_2 receptor-mediated cAMP production. It is possible that the VIP_2 receptor response here is mediated predominantly by an isoform of AC which is unresponsive to Ptx-sensitive G proteins; mammalian AC type II, for example, is not inhibited by $G\alpha_i$ or $G\alpha_o$ and is stimulated by $G\alpha_s$, PKC and $\beta\gamma$ subunits, dependent on coincident $G\alpha_s$ stimulation (for review see: Sunahara et al., 1996). After prolonged CTx treatment in order to cause down-regulation of G_s, and stimulation of cAMP production with forskolin, it was still not possible to demonstrate an inhibitory effect on AC stimulation mediated by the VIP_2 receptor. In fact a stimulation of cAMP production was observed, over a concentration range more typical of [^{3}H]IP production than cAMP production (see Figure 3.16). This could be the result of $\beta\gamma$ subunit stimulation of an isoform such as AC type II or simply an inadequate treatment with forskolin and cholera toxin resulting in a system which is still subject to G_s-mediated stimulation. Mastoparan, reported to be a somewhat selective activator of Ptx-sensitive G proteins (Higashijima, 1996) caused a clear inhibition of forskolin/cholera toxin-stimulated cAMP production

suggesting that the failure of VIP_2 receptor stimulation to inhibit forskolin/cholera toxin-stimulated cAMP production was due to a characteristic of the VIP_2 receptor (see Figure 3.17). The inability of the VIP_2 receptor to inhibit CTx/forskolin stimulated cAMP production (despite there being considerable evidence of its ability to activate PTx-sensitive G proteins) may be attributable to its activating different PTx-sensitive G proteins to those activated by mastoparan. Alternatively, it may be the concomitant activation of other G proteins, such as G_s, which affect the VIP_2 receptors inhibitory influence.

3.4.5 Summary

The VIP_2 receptor has been demonstrated to signal through two distinct signalling pathways in COS 7 cells; cAMP production and also, for the first time, IP production. Stimulation of adenylate cyclase mediated by this receptor appears to be PTx-insensitive, indicating that there is no significant tonic inhibition of cAMP production by PTx-sensitive G proteins. The VIP_2 receptor-mediated stimulation of adenylate cyclase desensitises within 15 minutes, as does the PACAP receptor-mediated stimulation. The VIP_2 and PACAP receptors have similar EC_{50}s for PACAP-38-evoked cAMP production although the VIP_2 receptor stimulates a smaller response (which may not be solely due to a difference in expression levels).

The VIP_2 receptor has been shown to stimulate [^{3}H]IP production by a partly PTx-sensitive mechanism, while the PACAP receptors utilise a PTx-insensitive mechanism and stimulate a larger response with a lower EC_{50} for PACAP-38-evoked [^{3}H]IP production. The VIP_2 and PACAP receptors clearly utilise different mechanisms to stimulate PLC. Both G_q and a member of the $G_{i/o/t/z}$ family of PTx-sensitive G proteins were found to be associated with the VIP_2 receptor. This suggests that the PTx-insensitive component of VIP_2 receptor-

mediated [^{3}H]IP production is attributable to G_q stimulation of PLC and that the PTx-sensitive G protein is involved in an additional stimulatory mechanism.

VIP_2 receptor-mediated [^{3}H]IP production is at least partially dependent on Ca^{2+} influx, which occurs through a channel that may be activated by the emptying of intracellular Ca^{2+} stores ($CRAC_i$). Berven et al. (1995) described a role for a PTx-sensitive G protein, G_{i2}, acting between the release of Ca^{2+} from the intracellular stores and the stimulation of Ca^{2+} influx through the channel in the plasma membrane. However, this G protein would presumably not be receptor-associated. The VIP_2 receptor has been shown to be associated with a PTx-sensitive G protein but this, of course, does not exclude the possibility of a PTx-sensitive G protein also being associated with the intracellular stores. The receptor-associated PTx-sensitive G protein may simply be involved in a direct interaction with an SK&F 96365-sensitive Ca^{2+} channel that is not an L-type channel, or the $\beta\gamma$ subunits from the PTx-sensitive G protein may directly activate PLC. The model presented by Murthy & Makhlouf (1994) involves the stimulation of both an effector enzyme and a Ca^{2+} channel by a PTx-sensitive G protein. Lepretre & Mironneau (1994) described a pathway which involved a PTx-sensitive G protein stimulating Ca^{2+} influx through an L-type Ca^{2+} channel as a result of activating PKC. Berven et al. (1995) found that the activation of $CRAC_i$ in human neutrophils involved a PTx-sensitive G protein (G_{i2}) at a point between the emptying of intracellular Ca^{2+} stores and Ca^{2+} influx. This mechanism was also described in hepatocytes by Wenzelseifert et al. (1996) who found the Ca^{2+} influx to occur through a non-selective cation channel. Unfortunately the present data do not allow us to discriminate between different models other than to exclude a role for L-type Ca^{2+} channels. The simplest model that could be suggested at this stage involves the stimulation of PLC (possibly more than one isoform) by $G_q\alpha$ and $G_{i/o}\ \beta\gamma$ subunits.

The resultant emptying of intracellular stores stimulates Ca^{2+} influx , which causes further stimulation of PLC.

Figure 3.1 Homologous displacement of [^{125}I]PACAP-27 binding to whole COS 7 cells expressing the rat VIP_2 receptor.

Binding experiments were carried out at 0^{o}C for 60 minutes. [^{125}I]PACAP-27 was present at a concenrtration of 6.15±0.20 pM. The data are expressed as means±SEM. n=9.

Figure 3.2 Concentration-response curve for VIP-evoked cAMP production mediated by the VIP_2 receptor.

A typical example from 4 experiments is illustrated. In this example the maximum stimulation value was 21.1±0.8 fold of basal control. The EC_{50} was 0.84±0.03 nM. The basal value was 12.44±1.73 pmol/ml. cAMP production was measured in the presence of 0.5 mM IBMX. The values are means±SEM. n=6.

Figure 3.1

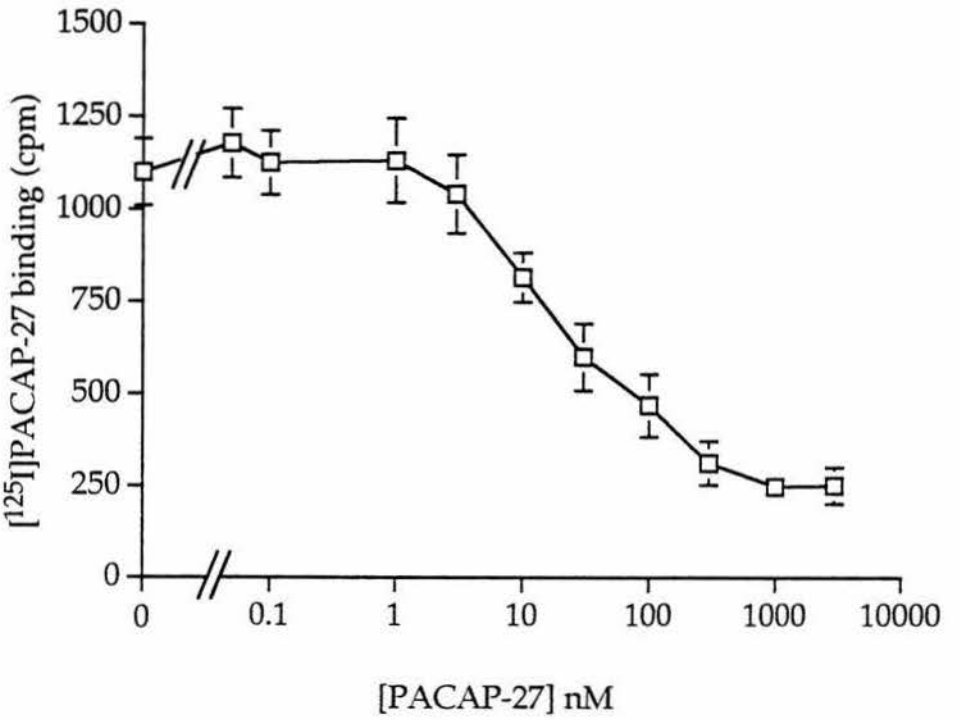


Figure 3.2

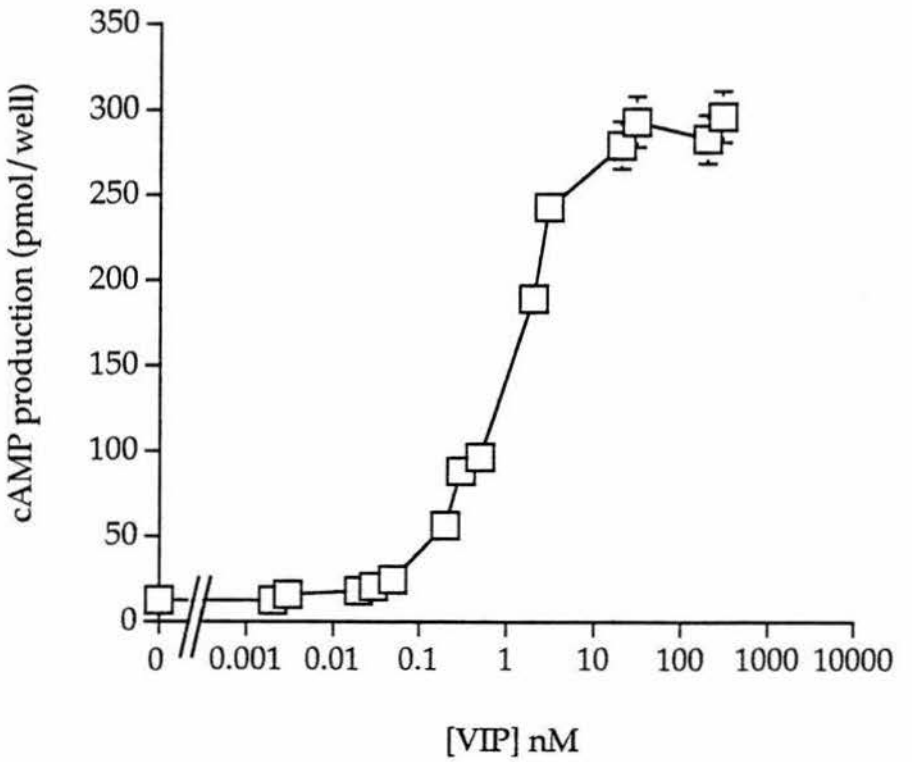


Table 3.1 Relative expression levels, IC_{50} values for homologous displacement of [^{125}I]PACAP-27, EC_{50} and maximal stimulation values (fold of basal control) for PACAP-38 and VIP-stimulated cAMP and [^{3}H]inositol phosphate production mediated by VIP_2, $PACAP_{short}$ and $PACAP_{long}$ receptors transiently expressed in COS 7 cells.

Bmax and IC_{50} values were determined by non-linear curve fitting of data from homologous displacement of ligand-binding experiments carried out on whole cells at 0°C for 60 minutes. EC_{50} values for [^{3}H]IP production were derived from non-linear curve fitting of concentration response curves. Values are expressed as means±SEM (n=6-9). Bmax is expressed as fmol/protein equivalent of 10^5 cells. EC_{50} is expressed in nM. The maximal stimulation (E_{max}) is expressed as 'fold of basal control'. A typical basal value for cAMP production was 6±2 pmol/well. A typical basal value for [^{3}H]inositol phosphate production was 7,700±600 dpm per well. EC_{50}s, IC_{50}s and Maximum Stimuli (E_{max}) values were derived from the non-linear curve-fitting programme P-fit (Elsevier Biosoft, Cambridge, UK).

Receptor	Specific [^{125}I] PACAP-27 Binding		cAMP Production				[^{3}H]IP Production			
			PACAP-38		VIP		PACAP-38		VIP	
	B_{max} (fmol/ 10^5 cells)	IC_{50} (nM)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)
VIP_2	59±5	19±1	0.9±0.1	7.8±0.5	0.3±0.1	8.2±0.1	36.9±8.1	3.0±0.3	45.0±6.6	2.4±0.1
$PACAP_{long}$	520±52	31±3	0.5±0.4	22.4±1.1	38.6±3.8	12.08±0.5	17.6±0.6	11.1±0.3		nd
$PACAP_{short}$	494±37	30±1	0.7±0.1	19.9±1.4	28.6±2.0	15.7±0.2	10.6±0.6	9.8±0.7		nd

(nd - no signal detected at doses up to 3 μM)

Figure 3.3 Concentration-response curve for VIP- and PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the VIP_2 receptor transiently expressed in COS 7 cells.

Typical examples from 4-6 experiments are illustrated. In this example VIP (□) caused a maximum stimulation was 2.36±0.22-fold of basal control. The EC_{50} value was 45.0±6.6 nM. For PACAP-38 (○) the maximum stimulation was 1.95±0.19-fold of basal control. The EC_{50} value was 56.5±8.9 nM. A typical basal value was 8,900±500 dpm/assay. The values are means±SEM. n=6.

Figure 3.4 Comparison of concentration-response curves for PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the $PACAP_{short}$ and $PACAP_{long}$ receptors transiently expressed in COS 7 cells.

Typical examples from 4 experiments are illustrated. In this example for the $PACAP_{short}$ receptor (○) the maximum stimulation was 9.1±0.3 fold of basal control and the EC_{50} value was 10.6±0.6 nM. A typical basal value was 6,340±56 dpm/assay. For the $PACAP_{long}$ receptor (▽) the maximum stimulation was 12.2±1.2 fold of basal control. The EC_{50} value was 17.6±0.6 nM. A typical basal value was 7,238±1,146 dpm/assay. The values are means±SEM. n=6.

Figure 3.3

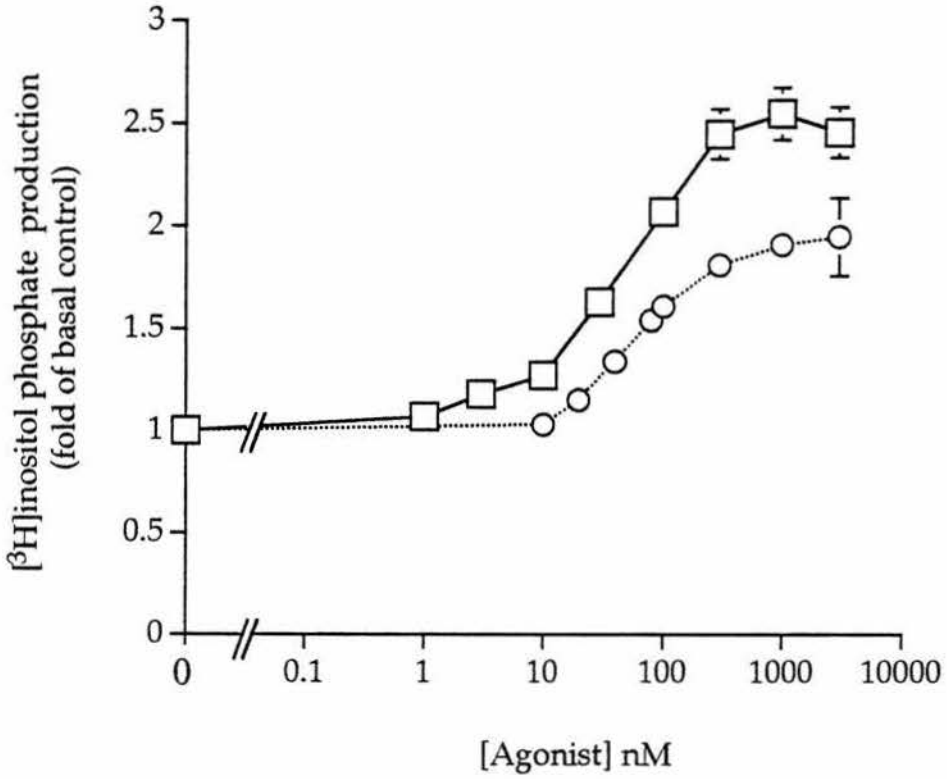


Figure 3.4

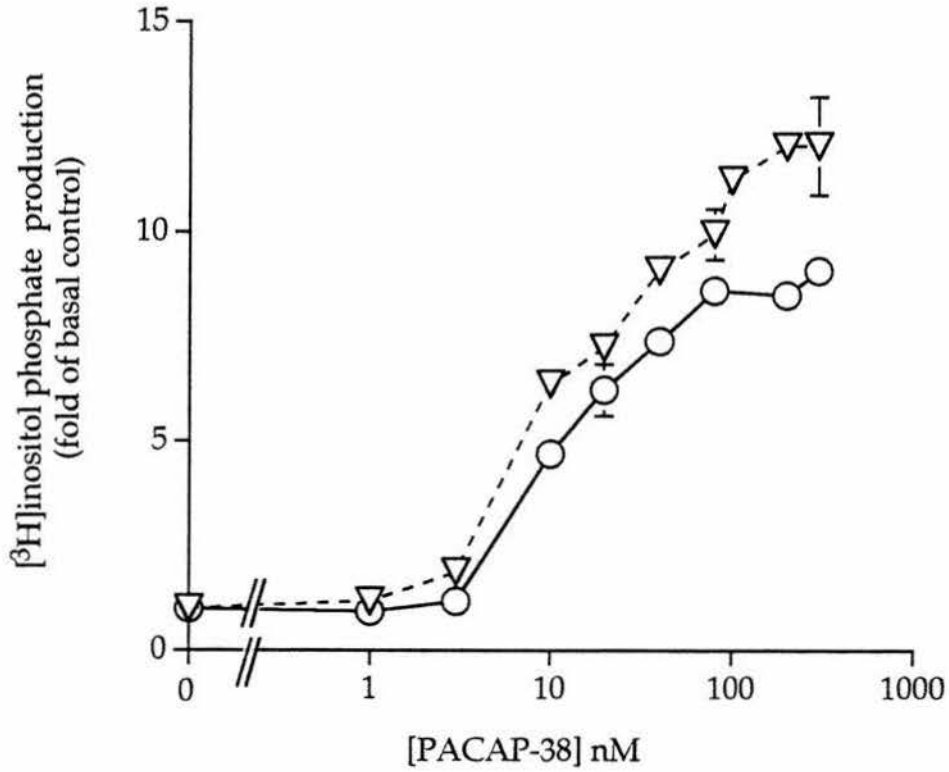


Figure 3.5 Time-course of VIP-evoked [^{3}H]inositol phosphate production mediated by the VIP_2 receptor transiently expressed in COS 7 cells.

The VIP (3 μM) -evoked stimulation after 80 minutes was 2.16±0.01 fold of basal control. A typical basal value was 4,910±43 dpm/assay. The values are means±SEM. n=6.

Figure 3.6 Time-course of PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the $PACAP_{long}$ receptor transiently expressed in COS 7 cells.

For the $PACAP_{long}$ receptor the 300 nM PACAP-38-evoked stimulation after 80 minutes was 7.31±1.04 fold of basal control. A typical basal value was 4,337±307 dpm/assay. The values are means±SEM. n=6.

Figure 3.5

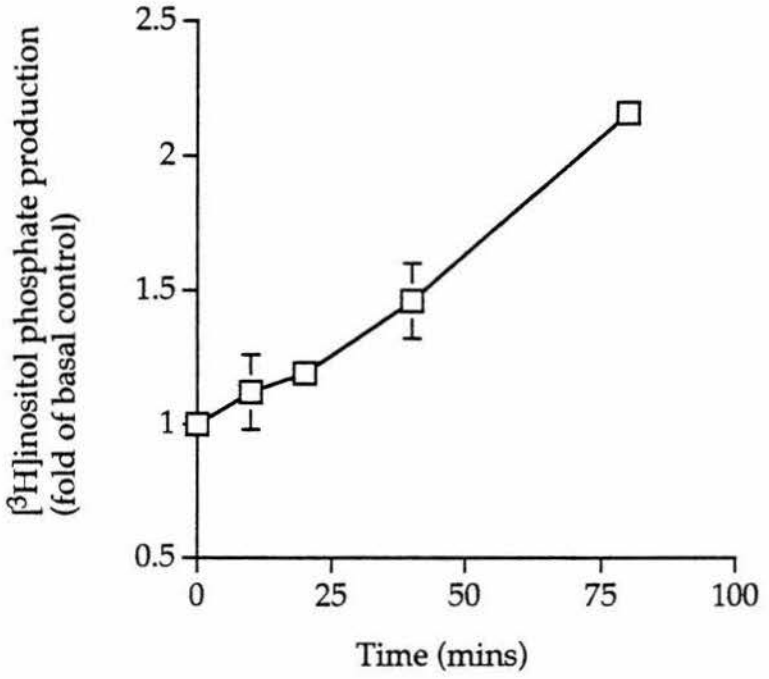


Figure 3.6

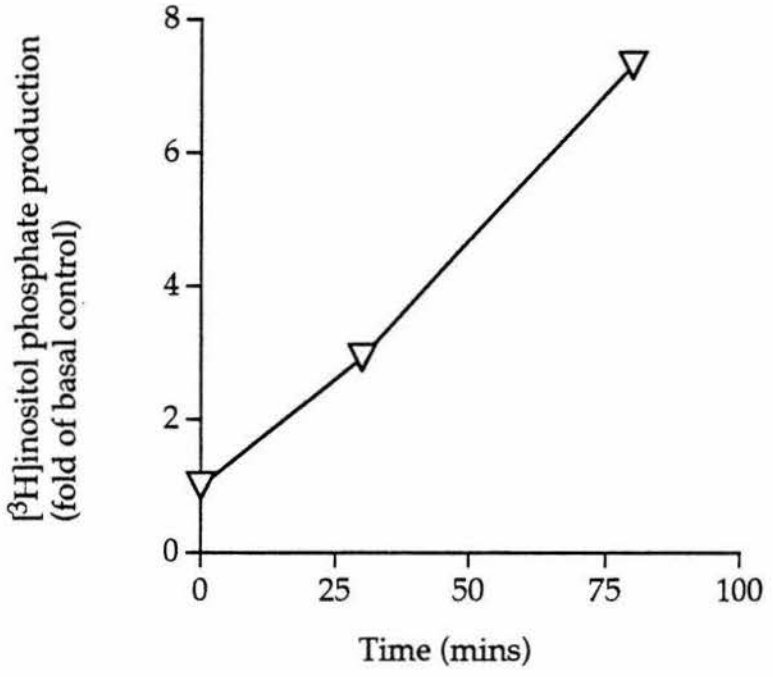


Figure 3.7 Time-courses for PACAP-38-evoked cAMP production mediated by the $PACAP_{short}$, $PACAP_{long}$ and VIP_2 receptors transiently expressed in COS 7 cells.

Intracellular cAMP accumulation was measured over 120 minutes. For the VIP_2 receptor (Δ) the stimulation after 15 minutes was 216±6 pmol/ml. A typical basal value was 9.9±0.7 pmol/ml. For the $PACAP_{short}$ receptor (◇) the stimulation after 15 minutes was 425.9±0.5 pmol/ml. A typical basal value was 8.1±0.5 pmol/ml. For the $PACAP_{long}$ receptor (□) the stimulation after 15 minutes was 396.8±8.3 pmol/ml. A typical basal value was 15.7±1.2 pmol/ml. cAMP production was measured in the presence of 0.5 mM IBMX. The values are means±SEM. n=6.

Figure 3.7

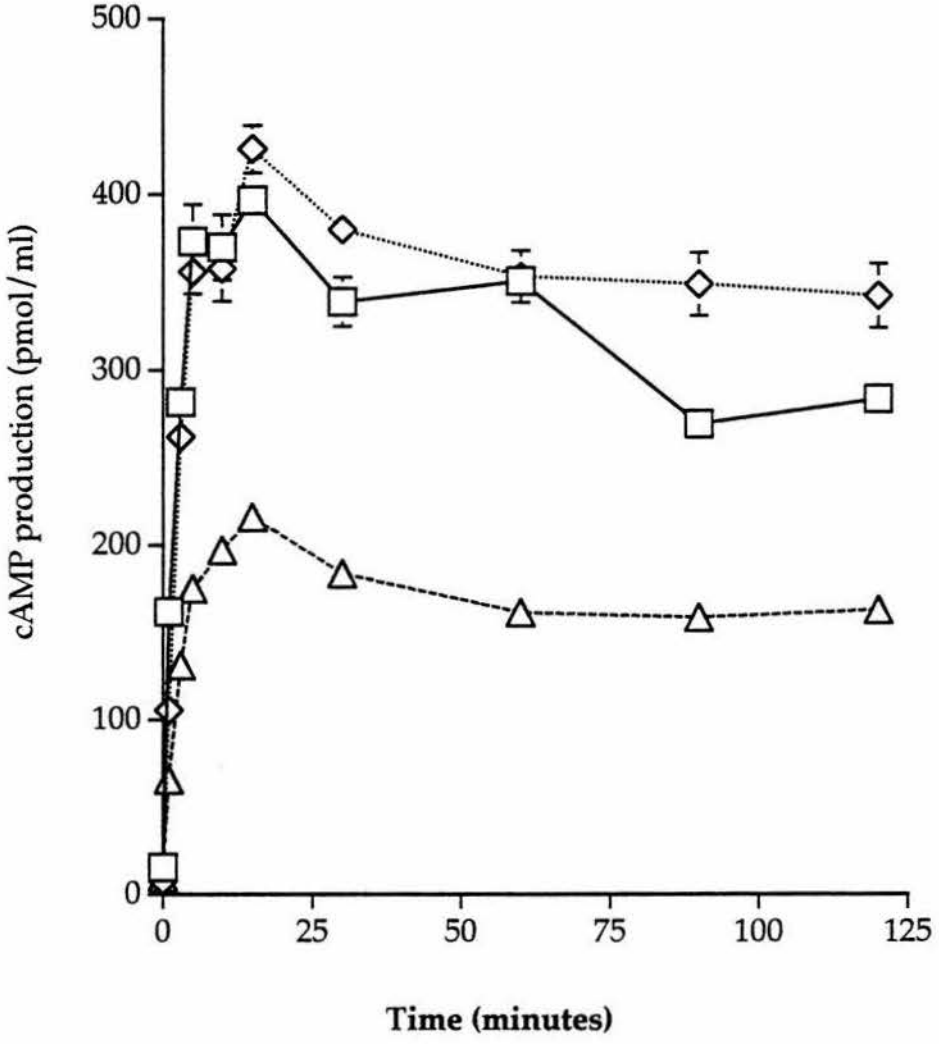

500
400
300
200
100
0
cAMP production (pmol/ml)
0
25
50
75
100
125
Time (minutes)

Figure 3.8

a) Pertussis toxin-sensitivity of VIP_1 and VIP_2 receptor-mediated [^{3}H]inositol phosphate production.

Typical basal activity was10,314±280 dpm/assay. Values are means ± SEM. n=10. There was no difference in basal [^{3}H]inositol phosphate formation in VIP_1 or VIP_2 receptor-expressing cells and data for these is expressed in a combined form. The maximum stimulation evoked by 3 μM VIP via the VIP_1 receptor was 2.55±0.16-fold of basal control was reduced to 1.84±0.09 fold of basal control after PTx-treatment. The maximum 3 μM VIP-evoked stimulation mediated by the VIP_2 receptor was 1.99±0.05-fold of basal control reduced to 1.61±0.10-fold of basal control after PTx-treatment. (*) represents a statistically significant inhibition of VIP-induced [^{3}H]inositol phosphate production ($p<0.05$ by Mann-Whitney *U*-test).

b) The effects of prolonged preincubation with a high concentration of cholera toxin and of pertussis toxin B-subunit on [^{3}H]inositol phosphate production elicited by the VIP_1 and VIP_2 receptors.

The control response to 3 μM VIP was typically around 29,869 dpm per assay. CTx or PTx B-subunit were present for 16 hours prior to stimulation. Values are means ± SEM. n=6.

c) Pertussis toxin-insensitivity of [^{3}H]inositol phosphate production mediated by the $PACAP_{short}$ and $PACAP_{long}$ receptor.

The maximum stimulation evoked by 100 nM PACAP-38 at the $PACAP_{short}$ receptor was 7.7±0.6-fold of basal control, 7.8±0.2-fold of basal control after PTx-treatment. The maximum stimulation evoked by 100 nM PACAP-38-evoked stimulation mediated by the $PACAP_{long}$ receptor was 10.6±0.8-fold of basal control, 11.0±0.3-fold of basal control after PTx-treatment. A typical basal value was 10,110±650 dpm/assay, 9,200±910 dpm/assay after PTx-treatment. Values are means ± SEM. n=6.

Figure 3.8

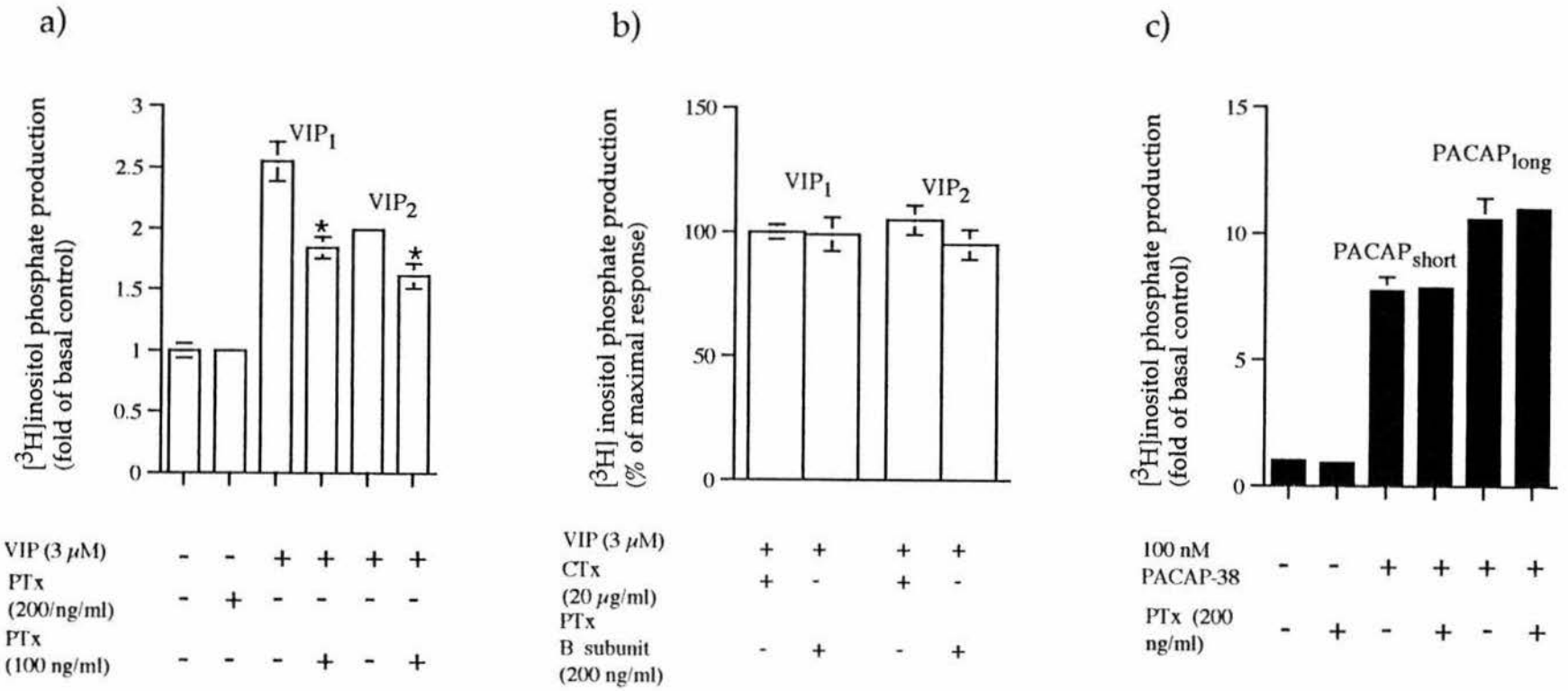

a)
[3H]inositol phosphate production (fold of basal control)
3
2.5
2
1.5
1
0.5
0
VIP1
VIP2
VIP (3 μM) - - + + + +
PTx (200/ng/ml) - + - - - -
PTx (100 ng/ml) - - - + - +
b)
[3H] inositol phosphate production (% of maximal response)
150
100
50
0
VIP1
VIP2
VIP (3 μM) + + + +
CTx (20 μg/ml) + - + -
PTx B subunit (200 ng/ml) - + - +
c)
[3H]inositol phosphate production (fold of basal control)
15
10
5
0
PACAPshort
PACAPlong
100 nM PACAP-38 - - + + + +
PTx (200 ng/ml) - + - + - +

Figure 3.9 The effect of pertussis toxin-pre-treatment on GTPγS-stimulated displacement of [^{125}I]helodermin binding to the VIP_2 receptor transiently expressed in COS 7 cells.

The assay was performed on a COS 7 cell membrane fraction. Where appropriate, cells were pre-treated with 100 ng/ml PTx for 16 hours. The maximum specific binding of [^{125}I]helodermin to the membrane preparation was equivalent to 13,344±252 cpm/assay for the control and 13,321±631 cpm/assay for the PTx treatment. The protein concentration of the membrane preparation was identical for the control and PTx-treated samples (100.8±4.1 and 100.4±14.0 µg/ml protein respectively). (*) represents a statistically significant reduction in the GTPγS -evoked effect on [^{125}I]helodermin binding by PTx-pretreatment (p<0.05 by Wilcoxon matched-pairs signed-rank test). The values are means±SEM. n=6.

Figur 3.10 Pertussis toxin-inhibition of mastoparan-stimulated [^{3}H]inositol phosphate production in COS 7 cells.

The open bars represent the mastoparan alone samples. The maximum response, stimulated by 15 µM mastoparan, was 1.34±0.08 fold of basal control. A typical basal value was 4,649±515 dpm/assay. The filled bars represent the PTx-treated samples. Ptx-treatment caused an mean inhibition of 46±11% of the mastoparan-stimulated response. Values are means ± SEM. n=6-9. (*) represents a statistically significant inhibition of mastoparan-stimulated [^{3}H]inositol phosphate production (p<0.05 by Wilcoxon matched pairs signed rank test).

Figure 3.9

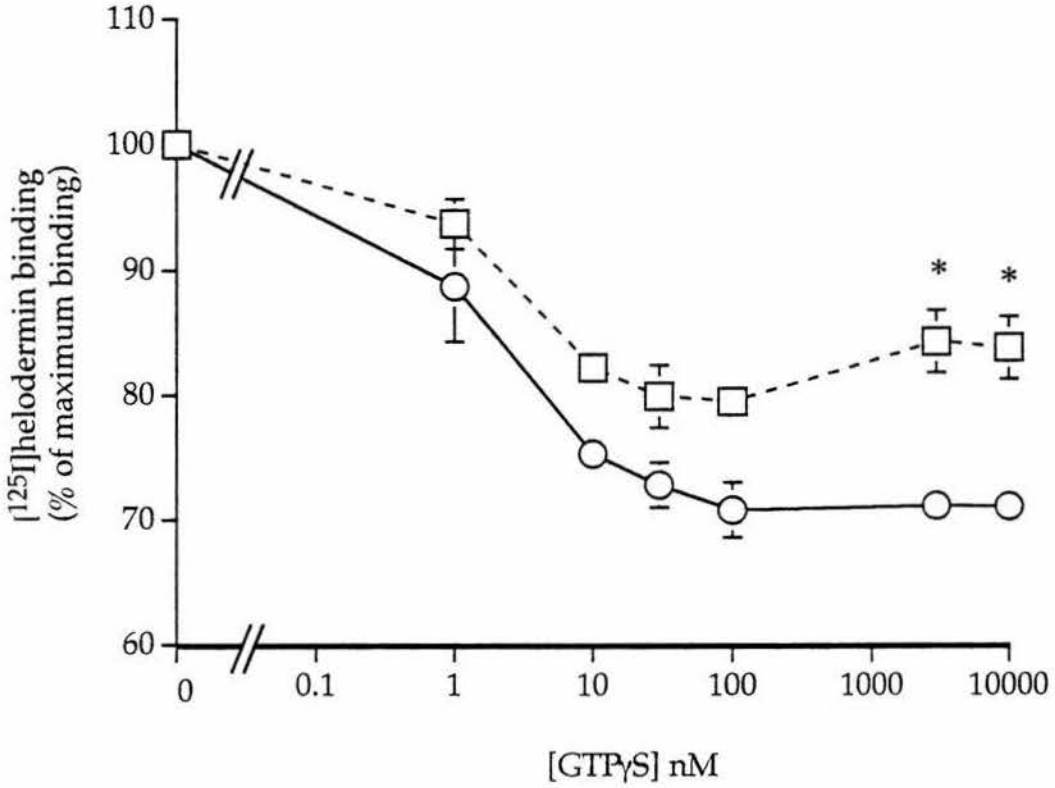


Figure 3.10

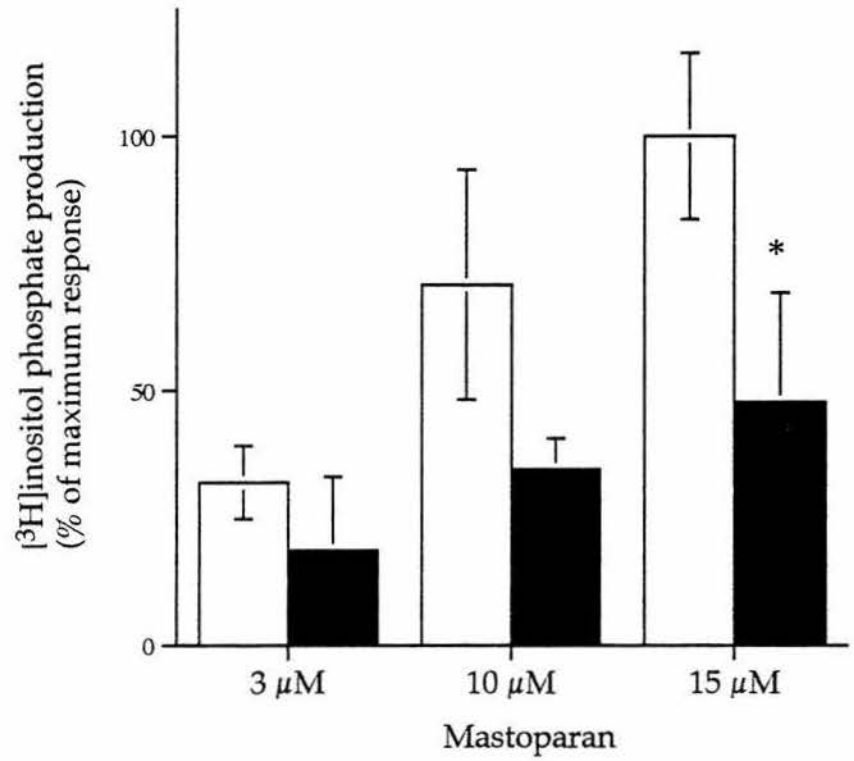


Figure 3.11 Concentration-response curve for ATP-evoked [^{3}H]inositol phosphate production in COS 7 cells.

The maximum stimulation was 93,380±255 dpm/assay. The EC_{50} value was 28.2±0.5 μM. A typical basal value was 2.837±121 dpm/assay. Stimulation of COS 7 cells with 100 μM ATP for 10 minutes caused no significant increase in cAMP production (84±29% of the basal control). The values are means±SEM. n=6.

Figure 3.11

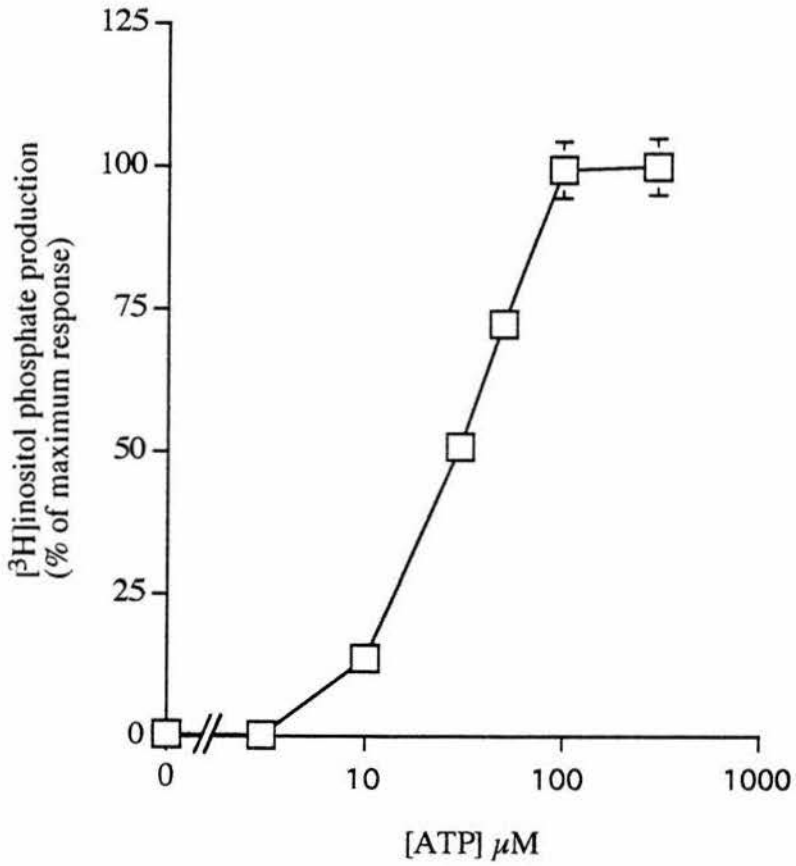

125
100
75
50
25
0
[3H]inositol phosphate production
(% of maximum response)
0
10
100
1000
[ATP] μM

Figure 3.12 The effects of Co^{2+}, methoxyverapamil and SK&F 96365 on [^{3}H]inositol phosphate production mediated by the VIP_2 and presumed P2Y receptors in COS 7 cells.

The control response for VIP_2 receptor-mediated activity (□) was the [^{3}H]IP production stimulated by 100 nM PACAP-38 (a typical response was 3.66±0.17 fold of basal control, a typical basal control was 3,778±318 dpm/assay). The control response for presumed P2Y receptor-mediated activity (O) was the [^{3}H]IP production stimulated by 50 μM ATP (a typical response was 13.9±0.1 fold of basal control, a typical basal control was 3,597±100 dpm/assay). The data are means±SEM. n=6-9.

a) the effects of the divalent cation channel blocker, Co^{2+}, on the [^{3}H]IP production mediated by the VIP_2 and presumed P2Y receptors.

b) the effects of the dihydropyridine(DHP)-sensitive Ca^{2+} channel antagonist, methoxyverapamil, on [^{3}H]IP production mediated by the VIP_2 and presumed P2Y receptors. Another structurally distinct antagonist of DHP-sensitive Ca^{2+} channels, nifedipine, had no inhibitory effect on the PACAP-38 stimulated response (120±13% of the control response after treatment with 10 μM nifedipine).

(c) the effects of the calcium-release activated calcium influx ($CRAC_I$) blocker and DHP-sensitive Ca^{2+} channel blocker, SK&F 96365, on the [^{3}H]IP production mediated by the VIP_2 and presumed P2Y receptors.

(*) represents a statistically significant inhibition of PACAP-38-induced [^{3}H]inositol phosphate production in comparison to the ATP-induced control response ($p<0.05$ by Mann-Whitney *U*-test).

Figure 3.12

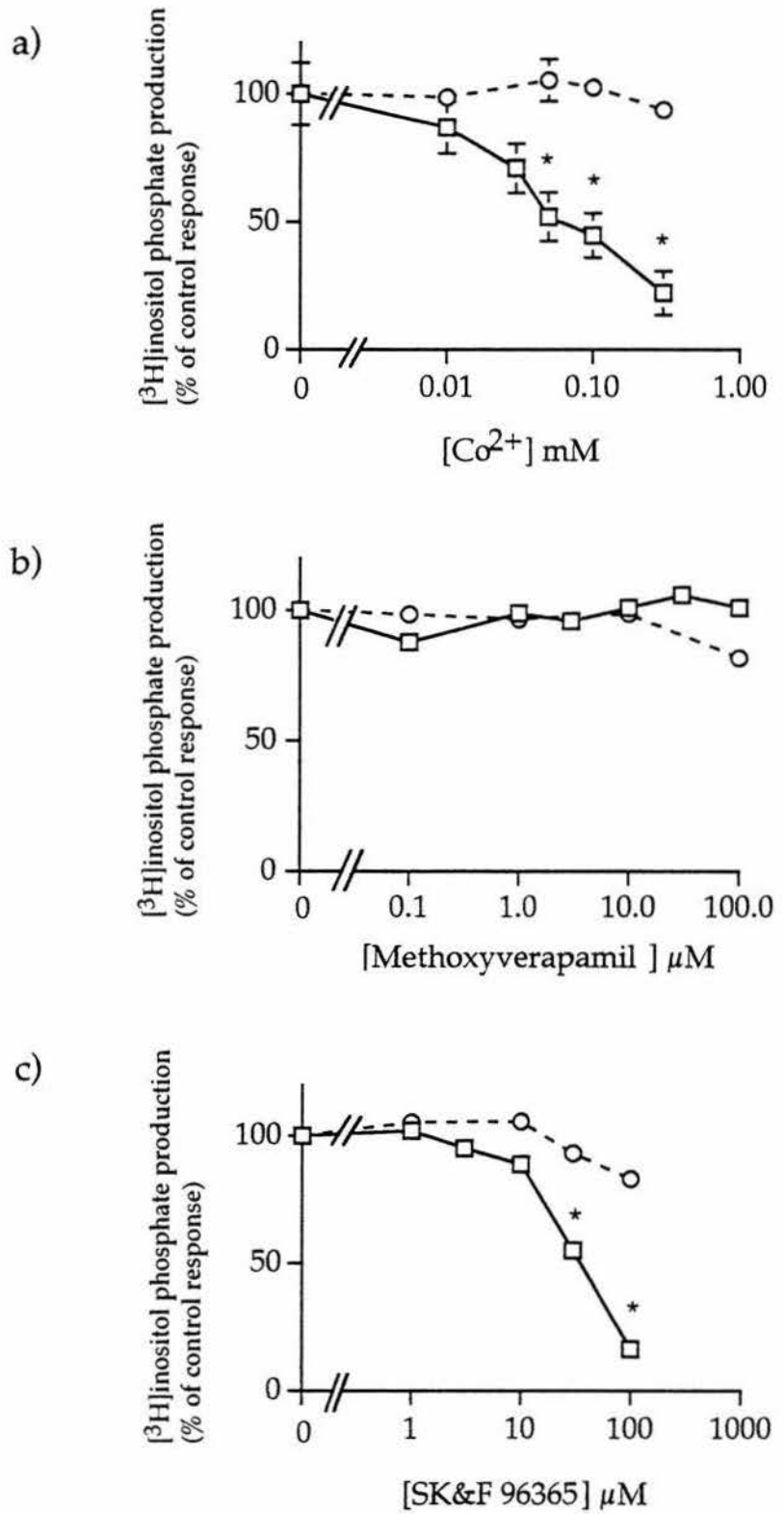

a)
[3H]inositol phosphate production
(% of control response)
100
50
0
0
0.01
0.10
1.00
[Co2+] mM
b)
[3H]inositol phosphate production
(% of control response)
100
50
0
0
0.1
1.0
10.0
100.0
[Methoxyverapamil] μM
c)
[3H]inositol phosphate production
(% of control response)
100
50
0
0
1
10
100
1000
[SK&F 96365] μM

Figure 3.13 The effect of thapsigargin treatment on basal and VIP_2 receptor-mediated [^{3}H]inositol phosphate production in COS 7 cells.

The control response was 8,658±493 dpm/assay. A typical basal value was 3,379±320 dpm/assay. (□) shows the effect of thapsigargin treatment on 100 nM PACAP-38-evoked [^{3}H]IP production. (O) shows the corresponding basal [^{3}H]IP production. The data are means±SEM. n=6. (*) indicates a statistically significant potentiation of 100 nM PACAP-38-evoked [^{3}H]IP production ($p<0.05$ by Mann-Whitney *U*-test).

Figure 3.14 The effect of Co^{2+} treatment on PACAP-38-evoked [^{3}H]inositol phosphate production in pertussis toxin-treated and control COS 7 cells expressing the VIP_2 receptor.

The control response stimulated by 100 nM PACAP-38 was 4,902±203 dpm/assay. (□) represents the 100 nM PACAP-38-evoked [^{3}H]IP production. (Δ) represents the 100 nM PACAP-38-evoked [^{3}H]IP production after PTx-treatment (100 ng/ml for 16 hours prior to stimulation with PACAP-38). The data are means±SEM. n=6.

Figure 3.13

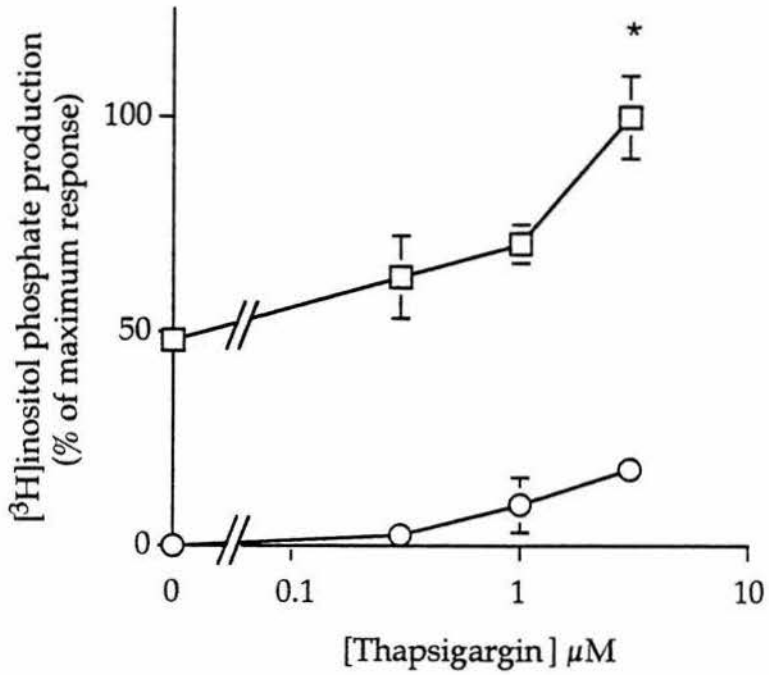


Figure 3.14

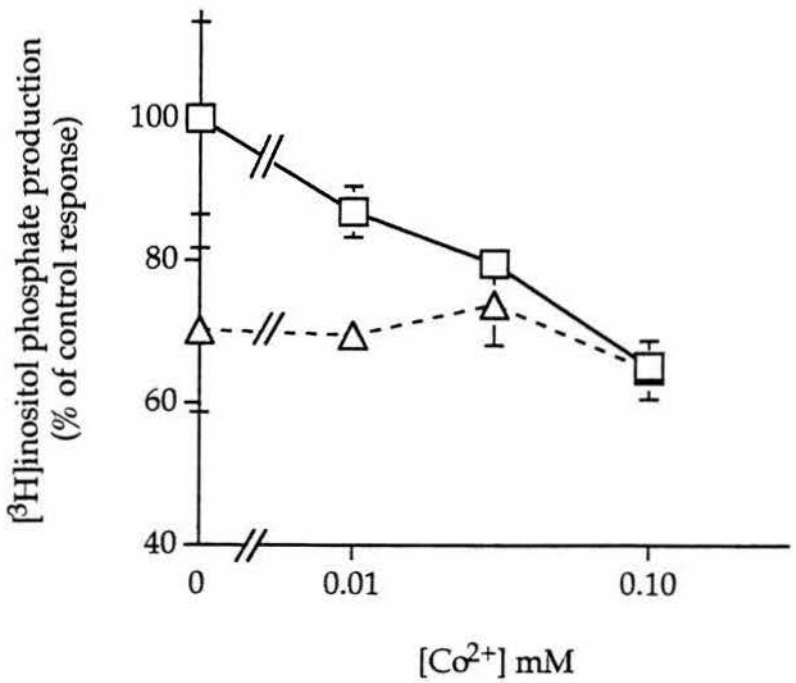


TABLE 3.2 The effect of Ca^{2+} channel blockers on $AlCl_3$/NaF -elicited [^{3}H]inositol phosphate production in COS 7 cells.

Treatment	Stimulation (%)
$AlCl_3$ (10 μM)/NaF (50 mM)	100.0±3.1
+ nifedipine (10 μM)	141.9±4.3 *
+ methoxyverapamil (30 μM)	131.8±5.4 *
+ SK&F 96365 (30 μM)	130.1±6.3 *

The 100% value represents a stimulation of 10.45 ± 0.49-fold of basal control. A typical basal value was 3,828±77 dpm per assay. The cells were preincubated with the inhibitors for 30 minutes before stimulation. The data are expressed as Means±SEM. n=6-9. (*) represents a statistically significant potentiation of $AlCl_3$/NaF-stimulated [^{3}H]inositol phosphate production ($p<0.05$ by Mann-Whitney *U*-test).

Figure 3.15 The effect of pertussis toxin-pretreatment on VIP-evoked cAMP production in COS 7 cells expressing the VIP_2 receptor.

Total cAMP accumulation was measured. At a concentration of 100 nM VIP the control response (open bars) was 106±4 pmol/ml, and the response from the PTx-treated sample (filled bars) 111±6 pmol/ml. There was no significant difference between the basal values for the control and PTx-treated samples, a combined basal value was 7.2±0.3 pmol/ml. PTx was applied at a concentration of 100 ng/ml for 16 hours prior to stimulation with VIP. The data are means±SEM. n=6.

Figure 3. 15

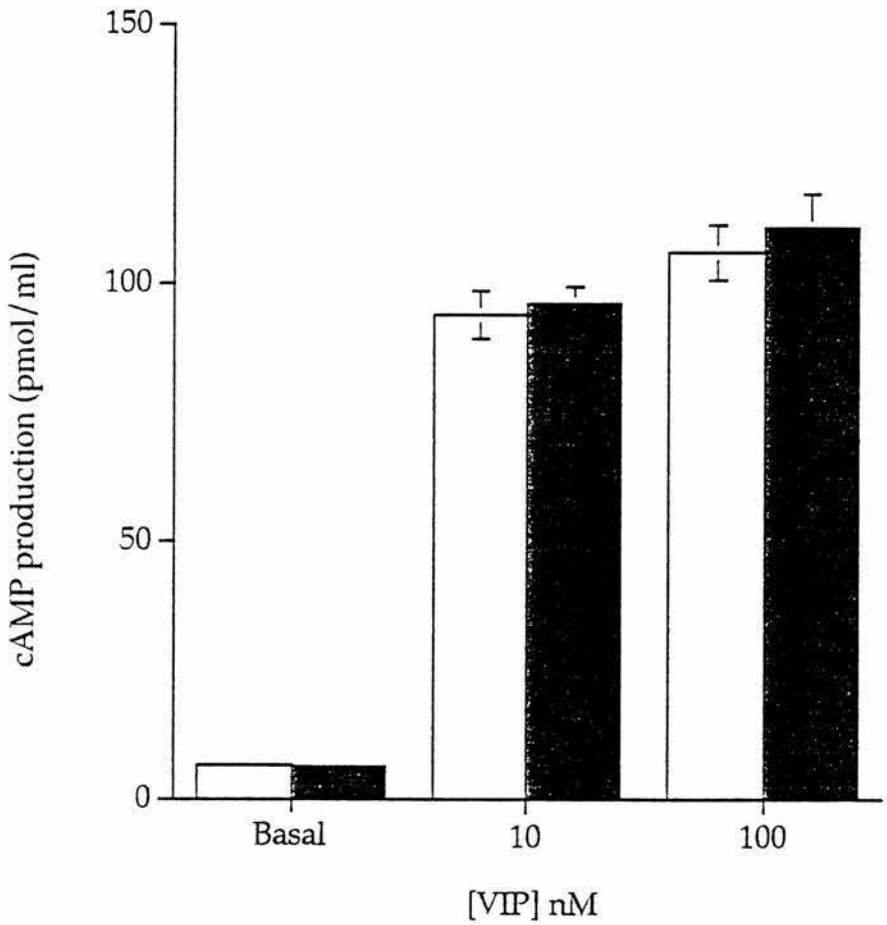


Figure 3.16 Concentration-response curve for PACAP-38-evoked cAMP production mediated by the VIP_2 receptor transiently expressed in COS 7 cells treated with forskolin and cholera toxin.

The cells were pre-treated with cholera toxin (20 μg/ml) for 16 hours prior to stimulation. Forskolin (10 μM) was added immediately prior to the addition of PACAP-38. A typical basal value was 1.11±0.02 pmol/ml. Forskolin alone stimulated cAMP production (to 101.0±3.9 pmol/ml) as did cholera toxin-pre-treatment (to 64.8±3.3 pmol/ml). cAMP production was measured in the presence of 0.5 mM IBMX. The values are means±SEM. n=6-9. (*) represents a statistically significant stimulation of cAMP production by PACAP-38 over that stimulated by a combined cholera toxin and forskolin treatment ($p<0.05$ by Mann-Whitney *U*-test).

Figure 3.17 The effects of cholera toxin, forskolin and mastoparan on cAMP production in COS 7 cells.

The control response (stimulated by cholera toxin and forskolin treatment) was 630±26 pmol/ml. The basal value was 2.0±0.2 pmol/ml, the PTx alone value was 2.3±0.1 pmol/ml. The accumulation of both intracellular and extracellular cAMP was measured. Where appropriate, cells were pre-incubated for 16 hours with 100 ng/ml PTx and/or CTx at 20 μg/ml. Forskolin (Fsk) was applied for the period of the assay at 10 μM. Mastoparan (Mast.) was also applied for the period of the assay at 8 μM. The values are means±SEM. n=6-12.

Figure 3.16

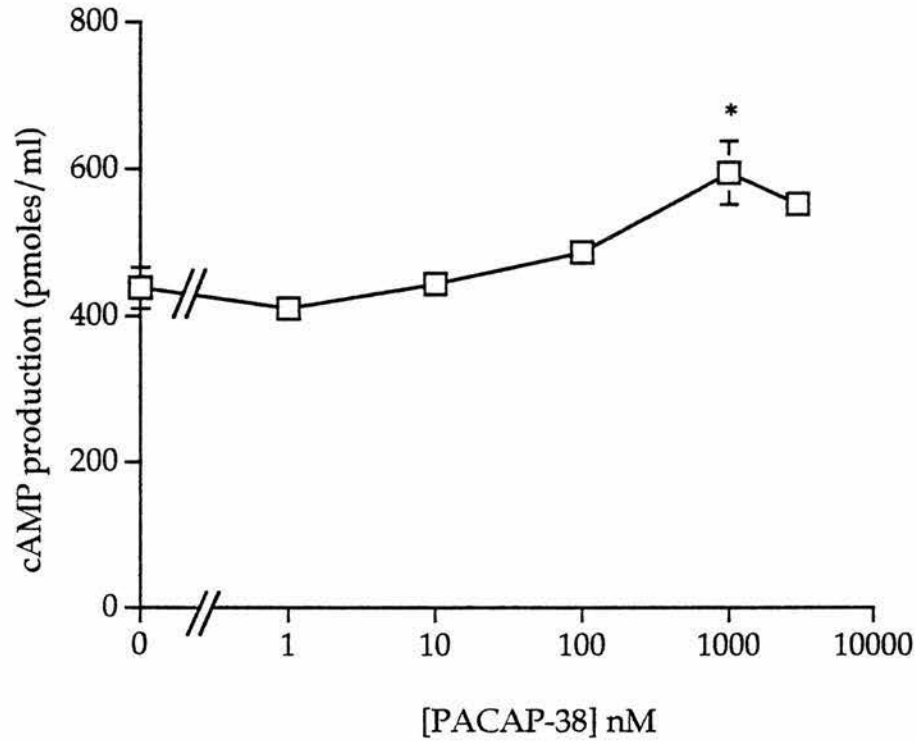


Figure 3.17

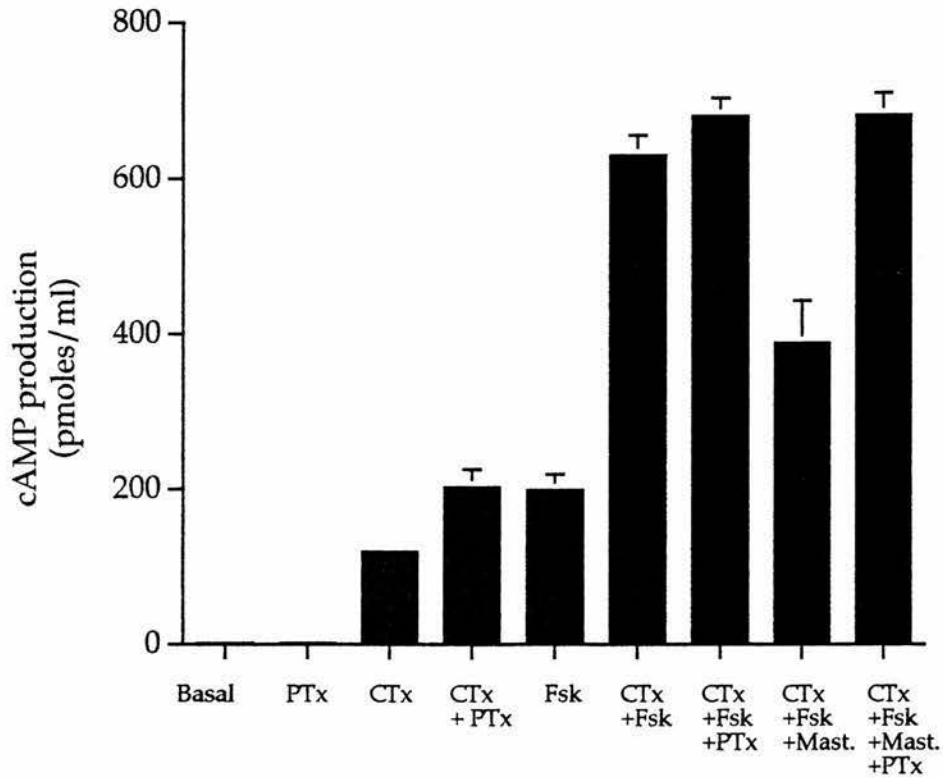


Table 3.3 The effect of various protein kinase inhibitors on [^{3}H]inositol phosphate production in COS 7 cells.

	[^{3}H]IP production (% of control response)			
	Expressed receptor			
	VIP_1	VIP_2	$PACAP_{long}$	*none*
	Agonist			
	VIP	VIP	PACAP-38	$AlCl_3$/NaF
Inhibitor	(1 μM)	(1 μM)	(300 nM)	(10 μM/50 mM)
GF 109203X (10 μM)	117.4±8.6	121.5±6.3	-	-
H89 (30 μM)	100.3±8.1	108.1±15.9	-	-
staurosporine (1 μM)	174.9±50.9	257.3±42.2	172.7±19.0	161.9±0.5
genistein (100 μM)	-	82.8±11.2	106.6±3.8	-

The PKC inhibitor, GF 109203X, the PKA inhibitor, H89, the broad spectrum kinase inhibitor, staurosporine and the tyrosine kinase inhibitor, genistein were all present for 10 minutes before stimulation and for the period of the assay. The combined basal value for the cells used in these experiments was 7,080±1,765 dpm/assay. The data are expressed as % of the control response (Means±SEM). A typical 1 μM VIP-evoked response at the VIP_1 receptor was 2.84±0.19-fold of basal control, at the VIP_2 receptor this was 2.03±0.04 fold of basal control. For the $PACAP_{long}$ receptor a typical 300 nM PACAP-38-evoked response was 7.74±0.63-fold of basal control. A typical 10 μM $AlCl_3$/50 mM NaF-evoked response was 13.4±0.3-fold of basal control. n=6-9.

Fig. 3.18 Concentration response curve for PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the HA epitope-tagged human VIP_2 receptor.

The receptor was transiently expressed in COS 7 cells. The maximum stimulation was 1.73±0.10 fold of basal control. The EC_{50} vale was 66.7±9.7 nM. A typical basal value was 4,340±308 dpm per assay. The data are means±SEM. n=6.

Figure 3.18

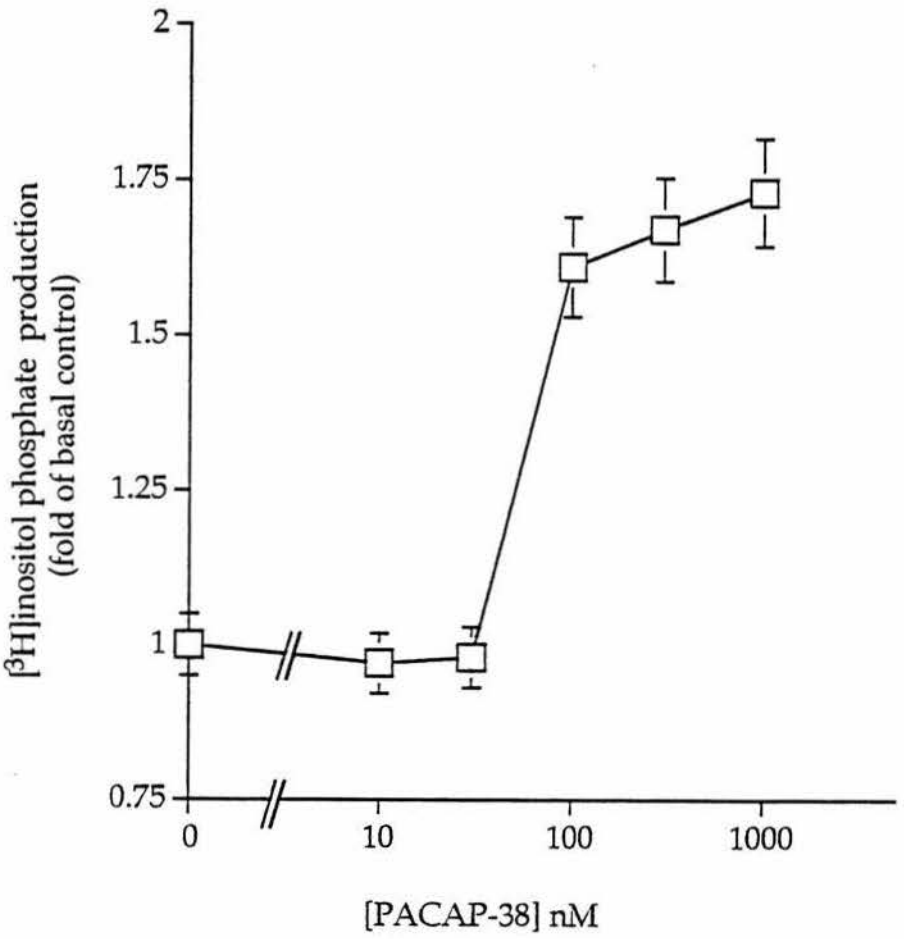
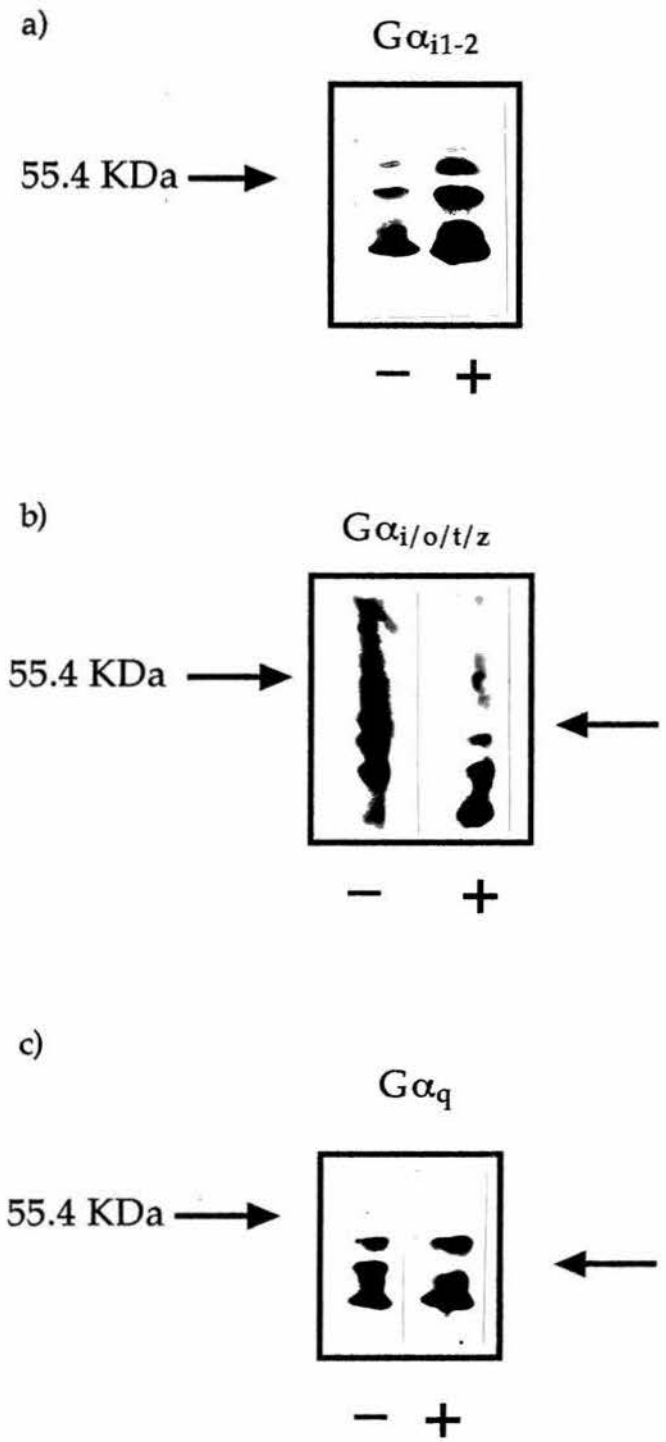

2
1.75
1.5
1.25
1
0.75
[3H]inositol phosphate production
(fold of basal control)
0
10
100
1000
[PACAP-38] nM

Figure 3.19 G protein α subunit isoform immunoreactivity associated with immunoprecipitates of the (HA) epitope-tagged human VIP_2 receptor.

The HA-tagged human VIP_2 receptor was transiently expressed in COS 7 cells and immunoprecipitated from a membrane preparation using an HA-tag-specific antibody. One dimensional SDS-PAGE followed by immunoblotting with G protein α subunit isoform specific antisera was carried out as described in sections 2.2.13 and 2.2.14. The left lane in each case (-) contains the sample immunostained with antiserum. The right lane in each case (+) contains the sample immunostained with antiserum preincubated with the specific peptide antigen. The left arrow indicates the position of the 55.4 kDa molecular weight marker. The right arrow identifies the position of any band specifically blocked by antigen treatment of the antiserum.

Figure 3.19



Chapter 4

Characteristics of truncated VIP_2 receptors and chimaeric VIP_2/PACAP receptors

4.1 Introduction

Chimaeric receptors created from members of the secretin/calcitonin/PTH/PTHrP receptor family have been used successfully in a number of studies to investigate the ligand-binding and signalling characteristics of specific receptors and to identify the contribution of specific functional domains to the receptors' activity. Studies have been carried out with , secretin/PTH/PTHrP receptor chimaerics (Turner et al., 1996; Lee et al., 1995), glucagon/GLP-I receptor chimaerics (Buggy et al., 1995) calcitonin/glucagon receptor chimaerics (Stroop et al., 1995) and VIP/secretin receptor chimaerics (Vilardaga et al., 1995; Holtmann et al., 1995; Gourlet et al., 1996).

This strategy has not previously been applied to the VIP and PACAP receptors despite the similarity in sequence of these receptors and the distinct and defined differences in both agonist pharmacology and agonist-induced second messenger production (which were described in Chapter 2). The receptors display a high degree of identity at the amino acid level (Table 4.3) and an even higher degree of similarity at the level of predicted secondary structure. This similarity should allow for the exchange of equivalent regions of receptor without the introduction of structural incompatibilities which are likely to affect the expression and function of the chimaeric receptors. The exchange of large regions of receptor should also involve a change in sequence of only a few residues, which means that important motifs and residues can be identified without the investment of time and resources involved in site-directed mutagenesis.

Our current understanding of the structure of G protein-coupled receptors is based on studies of the rhodopsin receptor for which a low resolution structure has been defined (Schertler et al., 1993; Unger & Schertler, 1995; Schertler et al., 1995).

The seven transmembrane-spanning domains of the rhodopsin receptor constitute an aspect of the receptors' structure predicted to apply to all G protein-coupled receptors (Baldwin, 1993; 1994). Indeed, a hydropathy plot of a cloned G protein-coupled receptor's amino acid sequence invariably reveals areas of hydrophobicity compatible with its having seven transmembrane-spanning regions.

Studies with chimaeric receptors have shown IC2, the N- and C-terminal portions of IC3 and the N-terminal portion of the C-terminus as being important for determining G protein selectivity (Dohlman et al., 1991; Savarese & Fraser, 1992; Strader et al., 1994; Hedin et al., 1993). Site-directed mutagenesis (SDM) studies of the rhodopsin (Francke et al., 1992), muscarinic (Zhu et al., 1994), α_{1B}-adrenergic (Scheer et al., 1996) and V_2 vasopressin receptors (Rosenthal et al., 1993) identified an Arg residue conserved in most GPCRs at the TM3/IC2 boundary as being essential for G protein coupling whereas Iida-Klein et al., (1997) identified a basic residue in IC2 as being essential for G_q coupling of the PTH receptor. Similar studies of the rhodopsin (Francke et al., 1992) and muscarinic receptors (Kunkel et al., 1993; Högger et al., 1995) identified positively charged residues at the C-terminal portion of IC3 which were critical for efficient G protein activation and hydrophobic residues at the N-terminal end of IC3 in the M_3 receptor (Blin et al., 1995; Burstein et al., 1995; Suprenant et al., 1992) as being important for G protein-coupling selectivity. Stroop et al. (1995) used GLP-I/glucagon receptor chimaerics to identify a high affinity binding site for calcitonin in the N-terminus whereas SDM and deletion mutagenesis were used to identify the N-terminus and EC3 of the PTH receptor as being crucial for PTH binding (Gardella etal., 1996; Lee et al., 1994). SDM studies have also revealed a number of residues in the N-termini and EC loops of the human VIP_1 and VIP_2 receptors which influence VIP binding (Nicole et al., 1997; Gaudin et al., 1995; Couvineau et al., 1995).

Chimaeric VIP_2/PACAP receptors were therefore created with exchange sites in the TM1, TM3, TM5 and TM7 regions (see Figure 4.9) which allowed the swapping of domains implicated in other receptors as being important for G protein activation, agonist binding and desensitisation

The C-terminal tail of secretin/calcitonin/PTH/PTHrP receptor family members has been identified as having profound effects on their behaviour. It has been shown to affect the internalisation, signal transduction and ligand-binding affinity of the calcitonin receptor (Findlay et al., 1994), the ligand-binding affinity and expression of the glucagon receptor (Unson et al., 1995) and the expression, internalisation and G protein selectivity of the PTH/PTHrP receptor (Huang et al., 1995; Huang et al., 1995b; Schneider et al., 1994). C-terminally truncated VIP_2 receptors were therefore also created (see Figure 4.10) in order to investigate the role of the C-terminus in this receptor's ligand-binding and signalling characteristics.

4.2 Specific Methodology

Chimaeric and truncated receptors - The chimaeric and truncated receptors were designed and constructed in this laboratory by Dr. Eve Lutz. The chimaeric receptors were constructed by exchanging equivalent regions of the wild-type rat VIP_2 and PACAP receptors. As discussed earlier, two major isoforms of the PACAP receptor have been identified in mammalian tissue. The two predominantly expressed splice variants are the 'short' form of 467 a.a. and the 'long' form which contains a 28 a.a. insert in the predicted IC3 region designated the 'hop1' cassette by Spengler et al. (1993) (see Figure 1.9). As a means of identifying any functional differences between these isoforms of the PACAP receptor, both were used for creating the chimaeric receptors. A HincII restriction site conserved

(within TM1), TM3 exchange sites and C-terminal exchange sites (TM7) were created by overlap extension polymerase chain reaction (PCR) mutagenesis. This involved the use of specific cDNA primers to create the required receptor fragments with terminal junction sequences that allow them to anneal as appropriate. The product of PCR amplification of the clone was digested with restriction enzymes and the fragments separated by agarose gel electrophoresis to confirm that the correct sized fragments were produced. All the constructs were sequenced to confirm the identity of the chosen clones.

The wild-type receptors:

$VIP_{2\ wt}$ (1-437)

$PACAP_{wt\ short}$ (1-467)

$PACAP_{wt\ long}$ (1-495)

The TM1 chimaeric structures were:

V_1P_{long} - $VIP_{2\ wt}$ (1-125) and $PACAP_{wt\ long}$ (153-495)

V_1P_{short} - $VIP_{2\ wt}$ (1-125) and $PACAP_{wt\ short}$ (153-467)

P_1V - PACAP (1-152) and $VIP_{2\ wt}$ (126-437)

The TM3 chimaeric structures were:

V_3P_{long} - $VIP_{2\ wt}$ (1-209) and $PACAP_{wt\ long}$ (234-495)

V_3P_{short} - $VIP_{2\ wt}$ (1-209) and $PACAP_{wt\ short}$ (234-467)

P_3V - PACAP(1-233) and $VIP_{2\ wt}$ (210-437)

The TM5 chimaerics:

V_5P_{long} - $VIP_{2\ wt}$ (1-293) and $PACAP_{wt\ long}$ (319-495)

V_5P_{short} - $VIP_{2\ wt}$ (1-293) and $PACAP_{wt\ short}$ (319-467)

P_5V - PACAP(1-318) and $VIP_{2\ wt}$ (294-437)

The TM7 chimaeric structures were:

V_7P - $VIP_{2\ wt}$ (1-376) and PACAP(429-495)

$P_{long}7V$ - $PACAP_{wt\ long}$ (1-428) and $VIP_{2\ wt}$ (377-437)

$P_{short}7V$ - $PACAP_{wt\ short}$ (1-400) and $VIP_{2\ wt}$ (377-437)

Two C-terminal truncated $VIP_{2\ wt}$ receptor constructs were prepared. The 391 a.a. truncated receptor (construct 6.3T: $VIP_{2\ wt}$ (1-391)) was prepared by EcoRI digestion of the cDNA for the $VIP_{2\ wt}$ receptor followed by subcloning and sequencing of the fragments. A more severe truncation, $VIP_{2\ wt}$ (1–376), was prepared by PCR using a primer specific to a region in TM7 (see Figure 4.9).

The cDNA was ligated into pBluescript for selection of appropriate clones by sequence analysis then inserted into pcDNA1 (Invitrogen, R&D Systems Europe Ltd., Abingdon, UK) for functional expression in COS 7 cells. Grow up and transfection procedures were described in sections 2.2.2 and 2.2.3.

Expression of receptors - COS 7 cells were transiently transfected with cDNA encoding the receptors as described in sections 2.2.1, 2.2.2 and 2.2.3.

Ligand-binding - Homologous displacement of ligand-binding was carried out on membranes preparations using [^{125}I]VIP or [^{125}I]PACAP-27 at 37^{o}C for 10 minutes, or on whole cells at 0^{o}C for 60 minutes, as described in Section 2.2.7.

Protein assay - Coomassie protein assay reagent was used to determine the protein level in samples (see section 2.2.8).

Second messenger assays - Intracellular cAMP production over a 10 minute stimulation period was measured in whole cells by radioimmunoassay as described in Section 2.2.5.

[^{3}H]inositol phosphate production was used as a measure of PLC activity. The cells were normally stimulated for 60 minutes with agonist before separation of [^{3}H]inositol phosphate by anion exchange chromatography as described in Section 2.2.4.

Data analysis - Curve fitting was performed by the non-linear curve-fitting programme, P-fit (Elsevier Biosoft, Cambridge).

4.3 Results

4.3.1 Ligand binding studies to monitor expression levels of chimaeric receptors and their affinities for PACAP-27

In radioligand-binding experiments homologous displacement of [^{125}I]PACAP-27 from whole COS 7 cells (see 'Materials & Methods' section 2.2.7) was used to give an estimate of the receptor binding capacity of the system (B_{max}) and the affinities of the receptors for PACAP-27 (IC_{50}) using the method of Swillens (Swillens, 1992). PACAP-27 was used as opposed to PACAP-38 because it was the only PACAP and VIP_2 receptor agonist commercially available as a radioligand. The $PACAP_{wt\ long}$ receptor had a B_{max} of 520±52 fmol/10^5 COS 7 cells (see Table 4.1). The B_{max} for the $VIP_{2\ wt}$ receptor was only 11% of that for the $PACAP_{wt\ long}$ receptor although its affinity for PACAP-27 appeared to be higher (IC_{50} values of 19±1 and 31±3 nM respectively). The B_{max} for $PACAP_{wt\ short}$ was 95% of that for $PACAP_{wt\ long}$. The $P_{long}7V$ and $P_{short}7V$ chimaerics had expression levels of 59% and 44% of the $PACAP_{wt\ long}$ receptor respectively whilst having a slight but statistically significant increase in their affinities for PACAP-27 (IC_{50}s of 24±2 nM for $P_{long}7V$ and 16±1 nM for $P_{short}7V$ as opposed to 31±3 nM for the $PACAP_{wt\ long}$ receptor and 30±1 nM for $PACAP_{wt\ short}$). The P_5V chimaeric with the $VIP_{2\ wt}$ receptor body C-terminal to TM5 exchange site had a slightly lower expression level than the P_7V chimaerics (39% of the $PACAP_{wt\ long}$ value as opposed to 59% or 44%) . The P_3V chimaeric which has the $VIP_{2\ wt}$ receptor body C-terminal to the TM3 exchange site had a

B_{max} value of 234±57 indicating that it was expressed at similar levels to P_7V and P_5V chimaerics. The affinity of the P_3V receptor for PACAP-27 was however significantly reduced with an IC_{50} of 69±17 nM. The P_1V chimaeric has the $VIP_{2\ wt}$ receptor body with the PACAP receptor N-terminus. This receptor displayed a mean B_{max} value 25% of the $PACAP_{wt\ long}$ receptor value, indicating a further drop in expression level from the P_7V, P_5V and P_3V chimaerics. The affinity for PACAP-27 at the P_1V chimaeric however was unchanged from that for the P_3V chimaeric (IC_{50}s of 69±17 and 70±17 nM respectively).

The V_7P chimaeric which has the PACAP receptor C-terminus was expressed at a similar level to the $VIP_{2\ wt}$ receptor and showed a similar IC_{50} for PACAP-27 (19±1 and 16±5 nM respectively). The V_5P_{long} and V_5P_{short} chimaerics (which have the PACAP receptor body C-terminal of the TM5 exchange site) had increased expression levels over the $VIP_{2\ wt}$ receptor (44% and 31% of the $PACAP_{wt\ long}$ receptor levels respectively) and reduced affinities for PACAP-27 (IC_{50}s of 58±4 and 38±4 respectively). In addition to the regions of the PACAP receptor contained in the V_5P chimaerics, the V_3P chimaerics also have the IC2/TM4/EC2 region of the PACAP receptor. The V_3P_{long} had a similar expression level (50% of the $PACAP_{wt\ long}$ receptor level) to the V_5P chimaerics whereas the V_3P_{short} chimaeric appeared to have a somewhat lower expression level at 18% of the $PACAP_{wt\ long}$ receptor level. The affinities of these constructs for PACAP-27 was not significantly different to that of the V_5P chimaerics with IC_{50}s of 72±10 nM and 56±14 nM for the V_3P_{long} and V_3P_{short} chimaerics respectively. The V_1P_{long} and V_1P_{short} chimaerics had expression levels of 29% and 41% of the $PACAP_{wt\ long}$ receptor level respectively, similar to the V_5P chimaeric levels. The affinity for PACAP-27 however was much higher than at the V_5P and V_3P chimaerics with V_1P_{long} and V_1P_{short} having IC_{50}s of 13±1 and 16±2 nM respectively.

4.3.2 Agonist-evoked cAMP Production by Chimaeric VIP_2/PACAP receptors

PACAP-38 stimulated cAMP production

Concentration-response experiments were carried out for each receptor construct, in which all of the chimaeric receptors displayed clear cAMP responses to the agonists PACAP-38 and VIP but with some distinctive differences. Based on previous experiments on wild-type receptors the cells were stimulated for 10 minutes with agonist. The EC_{50}s for PACAP-38 stimulated cAMP production lay mainly in the range 0.3-0.9 nM (see Table 4.1). The maximal stimulation values however showed more marked differences between the constructs. E_{max} values (expressed as fold of basal) for $P_{long}7V$ and $P_{short}7V$ receptors were unchanged with respect to the wild-type PACAP receptors. The P5V construct however displayed a maximal stimulation value approximately half of that observed for the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ receptors (10.5±0.5 as opposed to 22.4±1.1 or 19.9±1.4 fold of basal control respectively). The presence of the $VIP_{2\ wt}$ receptor C-terminal of TM5 appears to reduce the receptor's ability to stimulate adenylate cyclase. The P3V chimaeric values are very similar to those of P5V and P1V chimaerics. The $VIP_{2\ wt}$ receptor's maximum stimulation was even lower (7.8±0.5 fold of basal control). Interestingly the presence of the PACAP receptor's C-terminus in the V7P chimaeric increased the maximum stimulation by approximately 50% to 12.5±0.5 fold of basal control. The V5P chimaerics had similar maximum stimulation values to the V7P chimaeric whereas the corresponding values for the V3P constructs were 50% lower (6.3±0.1 and 7.0±0.3 fold of basal control for $V3P_{long}$ and $V3P_{short}$ respectively). The final chimaeric receptors in the series with the $VIP_{2\ wt}$ receptor N-terminus, $V1P_{long}$ and $V1P_{short}$ had EC_{50}s of 0.6±0.2 and 0.6±0.3 nM respectively and maxima even greater than

those for the wild-type PACAP receptors, 30.1±1.4 and 27.3±1.7 fold of basal control respectively. No marked differences were observed between the cAMP responses mediated by constructs containing equivalent regions of the $PACAP_{wt\ long}$ or $PACAP_{wt\ short}$ receptors.

The introduction of the regions of the $VIP_{2\ wt}$ receptor C-terminal to the TM5 exchange site into the PACAP receptor reduced its ability to stimulate adenylate cyclase. The PACAP receptor C-terminus increased the ability of the $VIP_{2\ wt}$ receptor to stimulate adenylate cyclase but the whole of the PACAP receptor body was required for the most efficient stimulation. The low E_{max} value displayed for the V_3P chimaerics is probably due to the disruption of important motifs proximal to the exchange site.

VIP-stimulated cAMP Production

Since the rat VIP_2 and PACAP receptors show characteristic differences in their affinities for VIP and PACAP-38 (the wild-type PACAP receptor being very poorly responsive to VIP in comparison to PACAP-38) the chimaeric receptor responses were also assessed using VIP as an agonist. The EC_{50}s for VIP-stimulated cAMP production cover a much broader range than those for PACAP-38. At the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ receptors the EC_{50}s for VIP-stimulated cAMP production were 38.6±3.8 and 28.6±2.0 nM, on average 59-fold greater than the EC_{50}s for PACAP-38. There was a similar relationship between the VIP and PACAP-38 EC_{50}s for the $P_{long7}V$ and $P_{short7}V$ chimaerics. The maximal responses for these receptors lay in the range 12-17 fold of basal control and were on average 72% of the maxima achieved with PACAP-38. The P_5V chimaeric had a maximum cAMP response approximately 30% of that for the wild-type PACAP receptors, a reduction was also observed for PACAP-38 stimulated cAMP production. The P_3V chimaeric had a VIP EC_{50} of 19.0±1.3 nM which was

significantly lower than the EC_{50}s determined for all the other N-terminal PACAP chimaerics. The maximum stimulation was also increased to approximately 50% of the average PACAP wild-type receptor maxima. The P_1V chimaeric had a greatly increased EC_{50} of 302.5±24.8 nM for VIP but a similar maximum stimulation of 8.3±0.1 fold of basal control which was 60% of the average maxima for PACAP wild-type receptors.

As would be expected, the EC_{50} for VIP-stimulated cAMP production at the $VIP_{2\ wt}$ receptor (0.3±0.1 nM) was much lower than at the PACAP receptors (38±3.8 and 28.6±2.0 nM at $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$). The maximum stimulation was 8.2±0.1 fold of basal control, very similar to that for PACAP-38. The $VIP_{2\ wt}$, V_7P, V_5P_{long}, V_5P_{short}, V_1P_{long} and V_1P_{short} receptors had similar EC_{50}s in the range 0.1-0.7 nM, the V_7P chimaeric receptor having the lowest EC_{50} (0.1±0.02 nM) and a maximum stimulation over 60% greater than that for the $VIP_{2\ wt}$ receptor (13.4±0.3 fold of basal control). The maximum stimulation levels were very similar to those for PACAP-38 stimulated cAMP production. An interesting exception is the V_3P chimaeric receptors which showed an increase in their EC_{50}s for VIP stimulated cAMP production (2.3±0.1 and 3.4±0.4 nM for V_3P_{long} and V_3P_{short} respectively) that was not observed for PACAP-38 stimulated cAMP production (0.2±0.1 and 0.5±0.1 nM respectively). As with the PACAP-38 stimulated maxima at the V_3P chimaerics, the VIP-stimulated maxima were much lower than at the V_7P, V_5P and V_1P chimaerics. The maximal VIP-stimulated cAMP responses at the V_3P chimaeric receptors were also approximately 33% lower than the maximum for PACAP-38 stimulated cAMP production. Therefore at these chimaeric receptors a reduced activity is observed for both peptides and a significant drop in potency for VIP but not PACAP-38. These effects can not be attributed to a low expression level since the $VIP_{2\ wt}$ and V_7P receptors are

expressed at much lower levels (B_{max} values of 59±5 and 46±15 fmol/10^5 cells respectively) than even the V_3P_{short} receptor (B_{max} of 91±24 fmol/10^5 cells).

As was observed for the PACAP-38 evoked cAMP responses, the PACAP receptor C-terminal tail increased the efficiency of VIP-evoked adenylate cyclase stimulation. However the whole PACAP receptor body was required for the most efficient stimulation. The TM3 exchange site in the V_3P chimaerics appeared to disrupt VIP-evoked cAMP production, as it did PACAP-38 evoked signalling, but to a greater extent since the EC_{50} for VIP was increased in addition to the maximal response being reduced. The P_3V chimaeric, whose PACAP-38 evoked signalling characteristics appeared unchanged from those for the P_5V and P_1V chimaeric receptors, displayed a reduced EC_{50} for VIP over that observed for all the other PACAP N-terminal chimaeric receptors.

4.3.3 Agonist-evoked [^{3}H]Inositol Phosphate Production by Chimaeric VIP_2/PACAP receptors

Concentration response experiments were carried out for all the receptor constructs using PACAP-38 and, in most cases, VIP as agonists:

PACAP-38 stimulated [^{3}H]inositol phosphate production

PACAP-38 stimulated [^{3}H]IP production occurred with similar EC_{50} values (10.6-17.6 nM) and maximum response levels (7.2-11.1-fold of basal control) for the wild-type PACAP receptors and the V_1P_{long} and V_1P_{short} chimaerics. The $P_{long}7V$ and $P_{short}7V$ chimaerics showed maximum levels of response approximately 50% lower than the wild-type PACAP receptors. This may be due to the difference in expression levels, B_{max} values of 520±52 and 494±37 fmol/10^5 cells for the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ receptors respectively, and 306±27 and 228±17 fmol/10^5 cells for the $P_{long}7V$ and $P_{short}7V$ receptors respectively.

The V_1P_{long} and V_1P_{short} chimaeric receptors however were expressed at lower levels (150±14 and 215±22 fmol/10^5 cells respectively) than the $P_{long}7V$ and $P_{short}7V$ chimaeric receptors yet show higher maximum levels of response (9.4±0.4 and 7.2±0.3 nM respectively). This suggests that it is the loss of elements in the PACAP receptor body that reduces the maximum level of response. The $P_{short}7V$ chimaeric receptor appeared to have an increased EC_{50} over the corresponding wild-type PACAP receptor (32.0±2.0 nM compared to 10.6±0.6 nM respectively). The P5V, P3V and P1V chimaerics had EC_{50}s in the range 43-56 nM; lower potencies for PACAP-38 than the wild-type PACAP receptors and the P7V chimaerics. The maximum stimulation by the P5V chimaeric was 29% , P3V was 14% and P1V mediated 9% of the maximum response of the $PACAP_{wt\ long}$ receptor (11.1±0.6 fold of basal control). The $VIP_{2\ wt}$ receptor had an EC_{50} of 36.9±8.1 nM and a maximum stimulation 29% of the wild-type PACAP receptor maximum . The V7P construct had a similar EC_{50} (40.6±3.6 nM) but a reduced maximum, 17% of that stimulated by the $PACAP_{wt\ long}$ receptor. The V5P chimaerics had EC_{50}s of approximately 59-64 nM and similar maxima to the V7P chimaeric (14.4-15.3% of the maximum response of the $PACAP_{wt\ long}$ receptor). Despite VIP and PACAP-38 stimulation of a cAMP signal at the V3P chimaerics no [^{3}H]IP signal was detected at concentrations up to 3 μM with either agonist.

The replacement of the PACAP receptor C-terminus with that of the $VIP_{2\ wt}$ receptor reduced the PACAP-38-evoked [^{3}H]IP stimulation, whereas chimaeric receptors containing sections of the $VIP_{2\ wt}$ receptor body (with the exception of the V3P chimaerics) displayed $VIP_{2\ wt}$ receptor-like EC_{50} and maximum stimulation values. The V3P chimaerics displayed an impaired ability to mediate agonist-evoked adenylate cyclase stimulation and this impaired signalling ability was also true for PACAP-38-evoked [^{3}H]IP production.

VIP-stimulated [^{3}H]inositol phosphate production

In section 4.3.2 VIP was shown to be considerably less potent than PACAP-38 for cAMP production mediated by the wild-type PACAP receptors and those chimaeric receptors which have N-terminal PACAP receptor sequence (PACAP-38 EC_{50} values were in the range 0.3 -1.4 nM whilst VIP EC_{50}s were in the range 19-303 nM). At the $VIP_{2\ wt}$ receptor and the N-terminal VIP_2 receptor chimaerics, with the exception of the V_3P chimaerics, VIP and PACAP-38 were shown to be of similar potency for cAMP production (0-4-0.9 nM for PACAP-38 and 0.1-0.7 nM for VIP) and to stimulate similar maximal responses (in the range 8-30 fold of basal control for PACAP-38 and 8-33 fold of basal control for VIP). Similarly, for [^{3}H]IP production, VIP was less potent than PACAP-38 at the wild-type PACAP receptors, to the extent that no [^{3}H]IP signal was detected at concentrations of VIP up to 3 μM while the PACAP-38 EC_{50}s were 17.6±0.6 and 10.6±0.6 nM for the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ receptors respectively. No VIP-evoked [^{3}H]IP signal was detected at concentrations of up to 3 μM at the P_7V and P_1V chimaeric receptors either, in view of this the P_5V and P_3V chimaeric reveptors were not examined for VIP-evoked [^{3}H]IP production. As suggested by the VIP-evoked cAMP production results VIP and PACAP-38 were of similar potency for $VIP_{2\ wt}$ receptor-mediated [^{3}H]IP production (EC_{50}s of 45.0±6.6 and 36.9±3.0 nM respectively). The V_1P chimaerics have only the N-terminal portion of the $VIP_{2\ wt}$ receptor but are capable of VIP-evoked [^{3}H]IP production as well as cAMP production. As described in section 4.3.2, VIP and PACAP-38 were equipotent and caused a level of maximal stimulation which was similar to that produced by the wild-type PACAP receptors. For [^{3}H]IP production at these chimaeric receptors VIP and PACAP-38 had similar potencies (15.5±1.3 and 31.3±0.6 nM for PACAP-38 and VIP respectively at the V_1P_{long} receptor and 15.7±2.8 and 20.3±1.3 nM for PACAP-38 and VIP respectively at the V_1P_{short} receptor). The

maximum stimulations all lay within the 7.2-10.3 fold of basal control range in which the maxima for PACAP-38-evoked [^{3}H]IP production by the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ also lay (11.1±0.3 and 9.8±0.7 fold of basal control respectively).

These results confirm that the equipotency for VIP and PACAP-38 associated with the $VIP_{2\ wt}$ receptor is a characteristic which can be attributed to the N-terminus of the receptor and that the disruption of domains proximal to the TM3 exchange site in the V_3P chimaeric may have been responsible for reducing the VIP and PACAP-38-evoked cAMP production and abolishing the receptor's ability to stimulate [^{3}H]IP production.

4.3.4 Pertussis toxin-sensitivity of [^{3}H]inositol phosphate production mediated by VIP_2 and PACAP chimaeric receptors

It was clearly demonstrated that the PACAP receptor mediates a non-PTx-sensitive stimulation of [^{3}H]IP production whereas the wild-type VIP_1 and VIP_2 receptors utilise a partially PTx-sensitive mechanism to stimulate PLC (see Chapter 3). In order to determine which of the functional domains in the VIP_2 receptor are responsible for conferring PTx-sensitivity, nine chimaeric VIP_2/PACAP receptor constructs (P_1V, P_3V, P_5V, $P_{long}7V$, $P_{short}7V$, $V5P_{long}$, $V5P_{short}$, $V7P$) were assayed for the PTx-sensitivity of their PLC responses (see table 4.2). 100 nM PACAP-38 was used to stimulate [^{3}H]IP production at each construct. The PTx-treated cells were pretreated for 16 hours with 100 ng/ml PTx before stimulation. As described in Chapter 3 the $VIP_{1\ wt}$ and $VIP_{2\ wt}$ receptor-mediated PLC responses were reduced to 55±3 and 59±5% of the corresponding control values after PTx-pretreatment. The $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ receptor-

mediated PLC responses were unaffected by PTx -pretreatment, being maintained at 101±3 and 104±3% of the control response. The [^{3}H]IP production responses of the P_1V, P_3V, P_5V and V_7P were significantly inhibited by PTx-pretreatment. However the V_5P_{long}, V_5P_{short}, $P_{long}7V$ and $P_{short}7V$ -mediated responses were entirely unaffected by PTx-pretreatment. This identifies the IC3/TM6/EC3 region of the $VIP_{2\ wt}$ receptor from the exchange site in TM5 to the site in TM7 (residues 293-375) as containing elements which are sufficient for the stimulation of PLC responses by a PTx-sensitive mechanism although the domains responsible for PTx-insensitive coupling to PLC cannot be discerned.

4.3.5 Characteristics of C-terminally Truncated VIP_2 Receptors

Ligand binding studies to monitor receptor expression levels and affinities for PACAP-27

A described in section 4.2.1 two C-terminally truncated $VIP_{2\ wt}$ receptors were created as a means of assessing the importance and role of the C-terminus in the expression of the $VIP_{2\ wt}$ receptor and its ability to mediate agonist-evoked second messenger production. As described for the chimaeric receptors earlier in this chapter, radioligand binding experiments involving the homologous displacement of [^{125}I]PACAP-27 were carried out in order to determine whether the receptors were being successfully expressed in the plasma membrane and if so at what level. These radioligand binding experiments were carried out on whole cells at 0^{o}C for 60 minutes. The three receptor constructs tested were, the $VIP_{2\ wt}$ receptor (VIP_2(1-437)), the truncated VIP_2 receptor which had a C-terminus of approximately 13 amino acids $VIP_2\Delta$(1-391) and the more severely truncated 376 amino acid construct $VIP_2\Delta$(1-376) which was truncated in the C-terminal portion

of TM7 (see Figure 4.10). The $VIP_{2\ wt}$ receptor was expressed in COS 7 cells with a B_{max} value of 113±22 fmol/10^5 cells and the $VIP_2\Delta(1\text{-}391)$ construct with a B_{max} value of 115±23 fmol/10^5 cells. The truncation of the C-terminal 45 amino acids therefore had no effect on the expression level of the construct in comparison to the wild-type receptor. Transfection of the $VIP_2\Delta(1\text{-}376)$ cDNA into COS 7 cells produced no detectable specific binding indicating that this construct was not expressed at the cells surface and that truncation of the $VIP_{2\ wt}$ receptor in the TM7 region probably disrupts the processing of the receptor within the cell and/or targetting of the construct to the cells surface.

The internalisation of receptors occurs at a negligible rate when binding studies are performed at 0°C. In order to study the internalisation of receptors a similar binding study was performed on whole cells at 37°C for 10 minutes (see 'Materials & Methods' Section 2.2.7). Homologous displacement of [^{125}I]VIP binding was carried out (see Figure 4.1). COS 7 cells transiently expressing the receptor constructs were prepared. Cells expressing the wild-type receptor $VIP_{2\ wt}$ and the 391 amino acid truncated receptor ($VIP_2\Delta(1\text{-}391)$) displayed similar affinities for VIP with IC_{50} values of 7.0±3.7 and 6.0±1.1 nM respectively. The B_{max} values were also similar, 0.19±0.04 and 0.11±0.03 pmol/mg protein respectively. As expected no specific binding was detected for the more severely truncated 376 amino acid receptor ($VIP_2\Delta(1\text{-}376)$).

Agonist-evoked cAMP production by wild-type and truncated VIP_2 receptors

The ability of the receptors to stimulate cAMP production was investigated in concentration-response and time-course experiments using VIP as agonist. Accumulation of intracellular cAMP was measured (see Figure 4.2). The concentration-dependence of VIP-evoked cAMP production mediated by the

$VIP_2\Delta(1\text{-}391)$ receptor was almost identical to that for the $VIP_{2\ wt}$ receptor with EC_{50}s of 0.12±0.03 and 0.18±0.03 nM respectively. Although it is not unusual for receptors which are expressed at levels too low to be detected by radioligand binding assays to mediate the stimulation of second messenger production, cAMP production in cells transfected with cDNA for $VIP_2\Delta(1\text{-}391)$ receptor remained at basal levels over the applied concentration range. This suggests that the receptor is expressed at negligible levels, if at all, since cAMP production is tightly coupled to the $VIP_{2\ wt}$ receptor. Because the C-terminal tail in a number of related receptors has been identified as modulating internalisation of the receptor and desensitisation of the second-messenger responses the time-dependence of the VIP-evoked cAMP accumulation by the wild-type and truncated receptors was examined. Figure 4.3(a) shows time-dependent accumulation of intracellular cAMP, Figure 4.3(b) shows the accumulation of total cAMP (intracellular and extracellular) and Figure 4.3(c) shows how the rate of accumulation changes over the 60 minute period for both the $VIP_{2\ wt}$ and $VIP_2\Delta(1\text{-}391)$ receptors. These data demonstrate that intracellular cAMP accumulation reached a plateau within five minutes for both receptors at between 20-25-fold of basal control. The accumulation of total cAMP did not appear to plateau for either receptor but the rate of accumulation decreases rapidly over the same period, as Figure 4.3(c) demonstrates. The rate of cAMP accumulation was maximal at approximately 1 minute and decreased rapidly thereafter. cAMP responses mediated by both receptors appeared to desensitise in the same way suggesting that the C-terminal tail of the $VIP_{2\ wt}$ receptor does not modulate cAMP production to any great extent.

Agonist-evoked [^{3}H]inositol phosphate production by wild-type and truncated VIP_2 receptors

The stimulation of [^{3}H]inositol phosphate production by the $VIP_{2\ wt}$ receptor is a less tightly coupled pathway than that for cAMP production and therefore one in which small changes in the receptor's ability to stimulate second messenger production may be more easily identified. Concentration response curves for VIP and PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the $VIP_2\Delta$(1-391) receptor were performed. Both VIP and PACAP-38 stimulated a concentration-dependent increase in [^{3}H]inositol phosphate production mediated by the $VIP_2\Delta$(1-391) receptor (Fig. 4.4 & 4.5). The EC_{50} value for VIP was 68.1±14.7 and for PACAP-38, 51.5±3.2, indicating that the peptides are equipotent at this receptor as they are at the $VIP_{2\ wt}$ receptor where corresponding values were 45.0±6.6 and 36.9±8.1 respectively. The maximum stimulation was 2.5±0.1 fold of basal control for VIP and 3.4±0.1 for PACAP-38. These values are also within the range observed for the $VIP_{2\ wt}$ receptor (2.4±0.1 and 3.0±0.3 fold of basal control respectively).

In order to investigate any possible effect that the C-terminal truncation might have on desensitisation of the PLC coupling by this receptor, the [^{3}H]IP production evoked by 1 μM VIP at $VIP_{2\ wt}$ and $VIP_2\Delta$(1-391) receptors was measured over an 80 minute period (Figure 4.6). The accumulation appeared to be linear for both receptors and there was no indication of desensitisation over the measured time-period, in striking contrast to the rapid desensitisation of cAMP responses described above (although the desensitisation behaviours may vary with the agonist dose). Since the wild-type VIP_2 receptor stimulates [^{3}H]IP production in a partially PTx-sensitive manner, experiments were carried out to investigate the

Ptx-sensitivity of this response at the $VIP_2\Delta(1\text{-}391)$ receptor and assess whether the C-terminus influences coupling to this pathway. Figure 4.7 shows a significant and comparable inhibition of both wild-type and truncated receptors stimulation of [^{3}H]IP production by PTx pre-treatment (100 ng/ml, 16 hours). There was no effect of Ptx on basal [^{3}H]IP levels and PTx pre-treatment reduced the $VIP_{2\ wt}$ -mediated response by 41±6% and the $VIP_2\Delta(1\text{-}391)$-mediated response by 31±6% . There is therefore no readily identifiable effect of the 45 amino acid truncation of the VIP_2 receptor C-terminus on agonist-evoked cAMP production or [^{3}H]IP production that is revealed by the concentration response curves for VIP or PACAP-38, the PTx-sensitivity of its [^{3}H]IP production or the time-course of its second messenger responses.

Although there was no evidence for differences in the endogenous desensitisation behaviour of the VIP_2 wt and $VIP_2\Delta(1\text{-}391)$ receptors, the C-terminus of the VIP_2 wt receptor contains a number of putative phosphorylation sites including consensus sequences for PKC phosphorylation. Thus the truncation could potentially lead to differences in the regulation of the receptor by kinases other than those involved in native desensitisation (e.g. PKC). The non-specific protein kinase inhibitor, staurosporine (Tamaoki et al., 1986) was used to identify any differences in the kinase sensitivity of the wild-type and truncated receptors (Figure 4.8). There was a significant potentiation of [^{3}H]IP production mediated by both receptors when staurosporine (1 μM) was present during the stimulation (from 1.8±0.15- to 3.49±0.4-fold of basal control and from 2.1±0.3- to 2.98±0.16-fold of basal control for the VIP_2 and $VIP_2\Delta(1\text{-}391)$ receptors respectively), this potentiation was also seen when the broad spectrum G protein activator $NaF/AlCl_3$ was used to stimulate [^{3}H]IP production (see Table 3.3) suggesting that the effect is downstream of the receptor and may be attributable to attenuation of the phosphorylation of a G protein or phospholipase C.

4.4 Discussion

4.4.1 Chimaeric VIP_2 and PACAP Receptors

Despite the homology between the wild-type PACAP and VIP_2 receptors there are considerable differences in their signalling characteristics. When transiently expressed in COS 7 cells the VIP_2 receptor mediates a modest stimulation of [^{3}H]IP production with a relatively high EC_{50} whereas the PACAP receptors mediate a larger magnitude response with an EC_{50} approximately 5-fold lower. The EC_{50}s for PACAP-38 stimulation of cAMP production are very similar at the PACAP and VIP_2 receptors although the maximum stimulation observed is two or more times greater at the PACAP receptors. However the most striking difference between the receptors is the poor stimulation of second messenger production by VIP at the PACAP receptor, where the EC_{50} for VIP-stimulated cAMP production is approximately fifty times greater than for PACAP-38. No detectable [^{3}H]IP signal was produced by VIP stimulation of the PACAP receptors within the concentration range tested here.

Pertussis toxin-sensitive PLC stimulation

The PACAP receptor stimulates PLC through the classical $G_{q/11}$ pathway which is PTx-insensitive, whereas the VIP_1 and VIP_2 receptors stimulate PLC through a PTx-sensitive mechanism (MacKenzie et al., 1996). Those chimaeric receptors which have the $VIP_{2\ wt}$ receptor sequence C-terminal of the exchange site in TM5 stimulate PLC in a PTx-sensitive manner (see section 4.3.4). Schneider et al. (1994) found that a C-terminally truncated PTH/PTHrP receptor expressed in HEK293 cells caused a partially PTx-sensitive stimulation of PLC, suggesting that the C-terminal tail restricted the interaction of the receptor with specific G proteins.

However in the $VIP_{2\ wt}$ receptor the C-terminal tail did not seem to influence the PTx-sensitive coupling, therefore, the major functional domain involved in the interaction with the PTx-sensitive G protein seems to be IC3 of the $VIP_{2\ wt}$ receptor (see Table 4.3). Iida-Klein et al. (1997) identified residues in IC2 of the rat PTH/PTHrP receptor as being required for G_q interaction although all three intracellular loops have been identified as being important for PLC activation by adrenergic, muscarinic and glycoprotein hormone receptors (O'Dowd et al., 1988; Chazenbalk et al., 1990; Dohlman et al., 1991; Savarese et al., 1992; Strader et al., 1994) with IC2 and IC3 being the most commonly implicated. The N-terminal and C-terminal portions of IC3 are predicted to be helical in structure and have been identified as important for G protein selectivity and activation. It is interesting to note that there a number of sequence differences between the VIP and PACAP receptors in this area, such as the arginine (Arg^{302} in the VIP_2 receptor) conserved in the VIP receptors yet substituted with a hydrophobic residue in the PACAP receptors.

Exchange of N-termini

By exchanging the N-terminus of the PACAP receptor with that of the $VIP_{2\ wt}$ receptor (V_1P), VIP and PACAP-38 were made equipotent for cAMP stimulation with a maximum that exceeded that of even the $PACAP_{wt}$ receptors. The V_1P construct was also capable of mediating VIP-stimulation of [^{3}H]IP production with a maximum response similar to that of PACAP-38 at the $PACAP_{wt}$ receptors and an EC_{50} of 20-30 nM whereas VIP-stimulated [^{3}H]IP production at the wild-type PACAP receptors was not detectable at concentrations up to 3 μM. The expression level of the V_1P construct was 2.5-3.5 times that of the $VIP_{2\ wt}$ receptor and 2.5-3.5 times lower than that of the $PACAP_{wt}$ receptors. VIP was slightly less potent than PACAP-38 for [^{3}H]IP production at this construct

(more marked for the $PACAP_{wt\ long}$ construct). It is possible that because PLC-coupling is much weaker than AC-coupling for these receptors, this difference is not apparent for agonist-evoked cAMP production. If the receptor number were reduced to the point that it became a limiting factor for AC stimulation, the difference might be observed for both pathways. It appears that the $VIP_{2\ wt}$ receptor N-terminus domain is sufficient to effect a major increase in VIP potency for both adenylate cyclase and PLC stimulation mediated by the $PACAP_{wt}$ receptors. The N-terminus domain must therefore contribute to the binding of agonist and/or its ability to induce an activated conformation of the receptor.

It has become clear through a number of studies on the secretin/calcitonin/PTH/PTHrP receptor family that agonist binding and activation of intracellular signalling cascades are separable functions and that both optimal binding and receptor activation involve multiple disparate elements in the receptor's sequence (Gourlet et al., 1996; Holtmann et al., 1995a; Holtmann et al., 1995b; Holtmann et al., 1996a; Holtmann et al., 1996b; Nicole et al., 1997; Knudsen et al., 1997; Turner et al., 1996). Studies on receptors of this family indicate that the N-terminus is one of the major sites of agonist binding and that peptide agonists have a distinct activation domain which tends to bind to motifs in the extracellular loops and transmembrane domains. Stroop et al. (1995) described a chimaeric receptor with the N-terminus of the calcitonin receptor fused to the glucagon receptor body which could bind salmon calcitonin (sCT) but could not mediate cAMP production as a result of the binding. The reversed construct with the N-terminus of the glucagon receptor fused to the calcitonin receptor body displayed no detectable binding of [^{125}I]sCT but did mediate the stimulation of cAMP production by sCT with an EC_{50} value of 2.2±0.6 nM. Their data indicated the presence of a high affinity binding-site in the N-terminus and an auxiliary low affinity activation site in the receptor body of the calcitonin receptor. Vilardaga et

al. (1995) created N-terminal exchange rat VIP_1 and secretin receptor chimaerics. It was found that those with the VIP_1 receptor N-terminus were capable of binding VIP with a similar IC_{50} to the wild-type VIP_1 receptor but with an EC_{50} for VIP-evoked cAMP production 15-fold greater. The same construct was poorly responsive to secretin having an EC_{50} for secretin-evoked adenylate cyclase activation 60-fold greater than the wild-type secretin receptor and an IC_{50} 5-fold larger. As a means of standardising receptor number the authors adopted the rather unusual strategy of downregulating expression of the receptors by a 24 hour incubation with agonist. Binding studies were then carried out at 25°C for the chimaeric receptors and 37°C for the wild-type. It was found that agonist binding to the chimaeric receptors occurred with a very short half-life and could be completely inhibited by the inclusion of 10 μM GTP in the assay. These authors were unable to demonstrate ligand-binding to the secretin N-terminal chimaeric receptor. The results are not easily interpreted beyond the point of saying that the VIP_1 receptor N-terminus appears to be sufficient to confer or improve VIP binding at the secretin receptor but not VIP-evoked AC activation. Gourlet et al. (1996) found that the rat VIP_1 receptor N-terminus reduced the EC_{50} for VIP-evoked adenylate cyclase activity at the secretin receptor by 10-fold. This value was still 100-fold greater than the EC_{50} for the wild-type VIP_1 receptor. In this study the EC_{50} for secretin-evoked adenylate cyclase activity for the VIP_1 receptor was unchanged by the addition of the secretin receptor N-terminus. In contrast Holtmann et al. (1995) found that the rat secretin receptor with the VIP_1 receptor N-terminus displayed wild-type VIP_1 receptor characteristics with respect to VIP binding and VIP-evoked adenylate cyclase activation. However, the EC1 region, in addition to the N-terminus of the secretin receptor was required in order to confer wild-type secretin binding and activation characteristics on the VIP_1 receptor. Holtmann et al. (1996) demonstrated that the rat secretin receptor was capable of

binding VIP with high affinity (K_i values for VIP and secretin were 0.7±0.2 and 2.1±0.5 nM respectively) but only secretin could stimulate AC activity with high potency (EC_{50} values of 0.5±0.1 and 83±12 nM for secretin and VIP respectively). The authors also created secretin and VIP_1 receptor fragments which consisted of the N-terminus, TM1 and a portion of IC1. These constructs were expressed in COS 7 cells and were found to bind VIP but not secretin (K_i values of 3.2±0.9 and 7.9±2.6 nM for the secretin and VIP_1 receptor constructs respectively). This is further evidence of a requirement for sequences in the secretin receptors trunk for secretin binding. Neither construct was capable of stimulating cAMP production. Cao et al. (1995) expressed a similar fragment of the rat PACAP receptor and found that this construct when expressed in COS 7 cells was a high affinity binding domain for PACAP with an affinity 20-times greater than the wild-type receptor. These studies suggest that for many members of this family of receptors, the major determinant of ligand binding is contained in the N-terminal portion of the receptor, as was observed for the $VIP_{2\ wt}$ and PACAP receptors in this study. A lower affinity binding site in the trunk of the receptor is implicated in the process of agonist-evoked AC activation.

In the present study the construct with the $PACAP_{wt}$ receptor N-terminus in the $VIP_{2\ wt}$ receptor body (P_1V) is expressed at more than twice the level of the $VIP_{2\ wt}$ receptor and is unable to mediate detectable VIP-stimulated [^{3}H]IP production. The VIP-stimulated cAMP production had an EC_{50} 100-times greater than that for the $VIP_{2\ wt}$ receptor and 10-times greater than for the $PACAP_{wt}$ receptors. The presence of the $PACAP_{wt}$ receptor N-terminus on the $VIP_{2\ wt}$ receptor body is therefore sufficient to attenuate VIP-stimulated signalling with respect to both cAMP and inositol phosphate responses with a much lesser effect on PACAP-38 potency in the same assays. The affinity of the receptor for PACAP-27 also seems to be reduced.

It is possible that the $PACAP_{wt}$ receptor contains a negative element for VIP binding/receptor activation. There are a number of cases now where negative elements for ligand-binding have been described. Turner et al. (1996) described a 'selectivity-filter' represented by a single residue near the extracellular surface of TM2 in the secretin and PTH/PTHrP receptors. An Asn192Ile mutation in the secretin receptor uncovered a response to PTH without affecting the secretin responses, the reciprocal mutation in the PTH/PTHrP receptor of Ile234Asn made the receptor responsive to secretin without affecting PTH responses. Pantaloni et al. (1996) have identified an additional splice variant of the PACAP receptor which is missing a 21 amino acid section of the N-terminus. In an earlier study these authors found PACAP-27 to be less potent than PACAP-38 at four splice variants of the rat PACAP receptor (which include the $PACAP_{wt\ long}$ and $PACAP_{wt\ short}$ splice variants used in this study) with respect to PLC stimulation (Spengler et al., 1993). The new splice variant does not display this characteristic, showing similar affinities for PACAP-27 and -38 and a greatly reduced EC_{50} for PACAP-27-evoked [^{3}H]IP production. This indicates that the 21 amino acid deletion may have removed a negative element for PACAP-27 binding; an 'exclusion sequence'.

The N-termini of the VIP and PACAP receptors contain six conserved cysteine residues and in the case of the VIP_1 and VIP_2 receptors all but one of these have been identified, through selective mutation studies, as being crucial for VIP binding (Couvineau et al., 1995; Nicole et al., 1997; Gaudin et al., 1995). A cysteine and a glutamate residue, which are not conserved in the PACAP receptors, have also been observed to be important for VIP binding at the human VIP_1 and/or VIP_2 receptors (see Table 4.3) (Nicole et al. 1997; Couvineau et al., 1995). The data clearly shows that the N-terminus is the major determinant of ligand-binding selectivity in these receptors but studies with receptors of the same family suggest that domains in the receptor body may also be involved in binding agonists.

Selective mutation studies have identified an aspartate residue and two cysteine residues in EC1 and a cysteine , tryptophan and threonine in EC2 of the human VIP_1 or VIP_2 receptors as being involved in binding VIP (Knudsen et al., 1997; Du et al., 1997; Nicole et al., 1997; Gaudin et al, 1995). All these residues are conserved for the VIP and PACAP receptors except for the threonine which is replaced with methionine in the PACAP receptors. These data are supportive of a two-site co-operation model of agonist binding in this receptor family, optimally requiring a second site in addition to the N-terminal domain.

Replacement of the PACAP receptor C-terminus by the $VIP_{2\ wt}$ C-terminus ($P_{long}7V$ and $P_{short}7V$) resulted in a halving of the maximal extent of PACAP-38 stimulated [^{3}H]IP production and an apparent increase in the EC_{50} for the $P_{short}7V$ chimaeric. VIP was ineffective on [^{3}H]IP production in both wild-type and chimaeric receptors. The reduction in PACAP-38-stimulated [^{3}H]IP production compared to wild-type PACAP receptors may be attributable to a drop in receptor number but this seems unlikely since the V_1P chimaerics, for example, are expressed at an even lower level yet mediate a PACAP-38 stimulated response almost twice as large. The potency and maximal extent of cAMP responses were little different in the P7V constructs with either PACAP-38 or VIP as agonist apart from a small apparent reduction in potency at the $P_{long}7V$ variant.

The reversed chimaeric, V7P, with the PACAP receptor C-terminus, has the lowest expression level of these constructs and a higher affinity for PACAP-27 in binding studies than most. With either PACAP-38 or VIP as agonist the EC_{50} for cAMP production appeared correspondingly to be slightly reduced compared to the wild-type VIP_2 receptor and the maximum increased by approximately 50%. [^{3}H]IP responses to PACAP-38 were unaltered. Thus it appears that the $VIP_{2\ wt}$ receptor C-terminal domain has little effect on PACAP receptor mediated cAMP

production but reduces the ability of the receptor to stimulate PLC. As was discussed earlier, the PACAP receptor stimulates PLC through a PTx-insensitive G protein, presumably $G_{q/11}$, whereas the $VIP2_{wt}$ receptor additionally utilises a PTx-sensitive mechanism involving a member of the $G_{i/o/z}$ family. The $VIP2_{wt}$ C-terminus could therefore have a negative influence on the ability of the receptor to interact with $G_{q/11}$. In contrast, the PACAP receptor C-terminus appears to improve the efficiency of the V7P construct compared to $VIP2_{wt}$ with respect to cAMP but not [^{3}H]IP responses.

The effect of $VIP2_{wt}$ receptor sequence on expression level

The exchange of the $PACAP_{wt}$ receptor C-terminus (residues 429-495) for that of the $VIP2_{wt}$ C-terminus (residues 376-437) appears to reduce the expression levels of the PACAP receptor-based chimeras. These are reduced further with the introduction of the $VIP2_{wt}$ TM5-TM7 region (residues 293-375) and further still with the $VIP2_{wt}$ TM1-TM5 region (residues 125-293). The exchange of the PACAP receptor N-terminus (residues 1-152) with the $VIP2_{wt}$ receptor N-terminus (residues 1-125), to form the $VIP2_{wt}$ receptor, causes a further reduction. This effect could be attributed to the removal of disparate elements which have a positive effect on expression level in the PACAP receptor sequence or the introduction of negative elements in the $VIP2_{wt}$ receptor sequence. The exchange of the $VIP2_{wt}$ receptor C-terminus for that of the PACAP receptor (V7P) has no effect on the $VIP2_{wt}$ expression level but the V5P chimaerics which have PACAP receptor sequence C-terminal of the exchange site in TM5 show a definite increase which is maintained in constructs with progressively greater C-terminal PACAP receptor components. These observations could be explained if the PACAP receptor C-terminus and portions of the TM5 to TM7 region co-operated in increasing expression levels of the receptors and the N-terminus and TM1 to TM3

region of the PACAP receptors also cooperated to increase expression levels. The replacement of the PACAP receptor C-terminus with that from the $VIP2_{wt}$ receptor reduced the expression level (P7V) whereas its addition to the $VIP2_{wt}$ receptor (V7P) had no effect. When both regions, PACAP receptor C-terminus (residues 429-425) and TM5-TM7 region (319-428), are present in the $VIP2_{wt}$ receptor (V5P) we see an increase in expression level is observed, as predicted. There was a further drop in expression level over that of P7V when the TM5 to TM7 region (residues 319-428) of the PACAP receptor was replaced with the equivalent region of the $VIP2_{wt}$ receptor suggesting that this region of the PACAP receptors may have had a small positive effect on expression level in the absence of the C-terminus. The further introduction of the TM1 to TM3 region (153-234) of the PACAP receptor (progressing from the V3P to V1P construct) had no effect on expression levels whereas its removal (progressing from the P3V to P1V construct) caused a reduction in expression levels. The drop in expression level in progressing from the P1V to $VIP2_{wt}$ receptor and increase from the V1P to $PACAP_{wt}$ receptors is supportive of there being cooperative positive elements in the N-terminus and TM1 to TM3 regions of the PACAP receptor. The argument against there being negative elements in the $VIP2_{wt}$ receptor dominating this process, as opposed to positive elements in the PACAP receptors, is simply that the replacement of the PACAP receptor C-terminus with the $VIP2_{wt}$ C-terminus reduced the expression level of the PACAP receptor (P7V) but the replacement of the $VIP2_{wt}$ C-terminus for the PACAP receptor C-terminus (V7P) had no effect on expression levels.

The role of the IC3/TM6/EC3 region of the receptor

The P_5V, P_3V and P_1V chimaerics all displayed $VIP_{2\ wt}$ receptor characteristics for PACAP-38 -stimulation of cAMP production, maximum stimulation being halved from $PACAP_{wt}$ and P_7V receptor levels. The introduction of residues 293 to 473 (TM5 to C-terminus) of the $VIP_{2\ wt}$ receptor into the $PACAP_{wt}$ receptor body (P_5V) caused a significant reduction in this receptor's ability to stimulate adenylate cyclase, without affecting its potency. Exchange of this region of the receptor also caused an increase in EC_{50} for PACAP-38 stimulated [^{3}H]IP production by P_5V and a drop in the maximum stimulation producing $VIP_{2\ wt}$ receptor-like IP signalling characteristics without the VIP responses. [^{3}H]IP production by the converse V_5P and V_1P chimaerics occurs by a PTx-insensitive mechanism (Table 4.2) whereas $VIP_{2\ wt}$- and V_7P -mediated stimulation was partially PTx-sensitive. VIP and PACAP-38 were equipotent for [^{3}H]IP production mediated by the V_5P chimaeric. The V_5P receptor stimulates [^{3}H]IP production through a PTx-insensitive mechanism as did the PACAP receptor, but with a much larger EC_{50} and a much lower maximum stimulation. This suggests that, as expected, there are multiple elements controlling the ability of the receptor to activate specific G proteins and therefore the whole of the PACAP receptor trunk appears to be required for optimal PACAP receptor-like PLC stimulation.

In addition, the data suggest that the TM5 to TM7 region of the PACAP receptor contains elements crucial for efficient interaction with G_s. Since in terms of maximal extent the most efficient stimulation of adenylate cyclase occurs via the V_1P chimaerics it appears that the whole PACAP receptor body and C-terminus are required for an optimal response. The TM7 regions are almost identical in

sequence except for Leu to Val, Cys to Gly and Leu to Phe substitutions in the PACAP receptor (see Table 4.3). The IC2 regions have little homology with each other; the PACAP receptor contains two basic residues and an acidic residue which are not present in the $VIP_{2\ wt}$ receptor. The TM5, TM6 and IC3 regions are very similar for both the $VIP_{2\ wt}$ and $PACAP_{wt\ short}$ receptors. There are several non-conservative substitutions however, the most striking of which are the Ile to Arg and Leu to Lys in IC3 of the $VIP_{2\ wt}$ receptor (see Table 4.3). No specific residues in these regions in the $VIP_{2\ wt}$ and PACAP receptors have been identified to date as being crucial to the receptors function. Key residues in the TM2 and TM7 regions of the PTH/PTHrP receptor have however been identified (Gardella et al., 1996; Turner et al., 1996). These residues are believed to facilitate an interaction between the TM2 and TM7 regions that has been proposed for rhodopsin family receptors (Henderson et al., 1990; Schertler et al., 1995; Suryanarayana et al., 1992; Sealfon et al., 1995; Zhou et al., 1994; Mizobe et al., 1996). The residues identified in TM2 are believed to form part of a polar face of a helix. The residues in question are all conserved in the VIP and PACAP receptors (i.e. Ser^{166}, Arg^{170} and Ser^{173} in TM2 and Gln^{365} in TM7 of the rat VIP_2 receptor).

The role of the EC2/TM4/IC2 region of the receptor

The EC_{50} for VIP-stimulated cAMP production at P_3V is the lowest of the N-terminal PACAP receptor chimaerics. The extracellular residues presented by the P_3V chimaeric may therefore present an improved environment for binding and activation by VIP over that for the other chimaerics with N-terminal PACAP receptor segments, particularly P_1V. PACAP-38-evoked cAMP and [^{3}H]IP production however appeared unchanged between the P_5V, P_3V and P_1V chimaerics. It is interesting that Holtmann et al. (1996) found that VIP did not bind to a chimaeric receptor consisting of the rat secretin receptor N-terminus with the

VIP_1 receptor body, yet bound with relatively high affinity to a construct containing the secretin receptor N-terminus and EC1 region in the VIP_1 receptor body. Many of the residues identified as being important for VIP binding by site-directed mutagenesis studies carried out on the human VIP_1 receptor (Knudsen et al., 1997; Nicole et al., 1997; Couvineau et al., 1995; Gaudin et al., 1995) and human VIP_2 receptor (Nicole et al., 1997) and are conserved in the PACAP receptors. The exceptions are Glu^{36} and Cys^{50} (equivalent to Glu^{23} and Cys^{37} in the rat VIP_2 receptor) identified in the N-terminus of the human VIP_1 receptor (Couvineau et al., 1995; Nicole et al., 1997). The data presented here emphasises the different binding requirements of agonists at the same receptor, and indeed the same agonist at different receptors, and the existence of separate binding and activation domains.

It is possible that the PACAP receptor contains a negative element for VIP binding as described for PACAP-27 at the PACAP receptor (Pantaloni et al., 1996) or PTH and secretin at the secretin and PTH/PTHrP receptors respectively (Turner at al., 1996). The data suggest that the TM3 to TM5 region (residues 209-293) of the $VIP_{2\ wt}$ receptor is particularly important for VIP binding/activation of signalling pathways. Its presence reduced the EC_{50} and increased the maximal extent of VIP- but not PACAP-38-stimulated cAMP production (P3V). Its absence (V3P) increased the EC_{50} and reduced the maximal stimulation for VIP-stimulated cAMP production whilst strikingly preventing stimulation of PLC by VIP or PACAP-38. The reduction in adenylate cyclase stimulation mediated by the V3P chimaeric and its inability to stimulate PLC in response to VIP or PACAP-38 suggests that cytoplasmic domains necessary for G_q stimulation are not present in the V3P chimaeric and some elements required for G_s activation are also missing or disrupted. Common elements for G protein activation and elements for activating specific G proteins may be affected. The TM3 exchange site occurs in a region that

is not completely conserved between the $VIP_{2\ wt}$ and PACAP receptors, the residues immediately N-terminal of the site are His for the PACAP receptor and Gln for the $VIP_{2\ wt}$ receptor. It therefore seems possible that the function of the TM3 domain may be disrupted affecting both agonist-receptor and receptor-G protein interactions. A network of functional connections between receptor domains could potentially be destroyed.

Functionally important residues, motifs, domains and interactions in G protein-coupled receptors

i) Important residues and motifs in the intracellular loops

Iida-Klein et al. (1997) identified a basic residue (Lys/Arg) at the C-terminal end of IC2 as being essential for G_q/PTH/PTHrP receptor interactions. Mutation of the GluLysLysTyr sequence to AspSerGluLeu abolished IP stimulation without affecting cAMP production. Mutation of the AspSerGluLeu motif to AspSerLysLeu or AspSerArgLeu was sufficient to restore the PLC response. Unfortunately the authors present no data on changes in EC_{50}s or expression levels caused by the different mutations only the effect on maximum stimulation levels. A basic residue is conserved at the equivalent position in the VIP and PACAP receptors (Arg^{237} in the rat VIP_2 receptor). A Leu which is conserved at an equivalent site in IC2 of the VIP and PACAP receptors is implicated in GnRH and m1 and m3 muscarinic receptor stimulation of PLC (Moro et al., 1993; Moro et al., 1994; Arora et al., 1995). A Phe residue in the β2-adrenergic receptor at the same relative position as Leu in the GnRH, m1 and m3 receptors is also important for isoproterenol-stimulated cAMP production (Moro et al., 1993). This suggests that the presence of a hydrophobic residue may be required at this position for G protein-coupling. The AspArgTyr motif identified in many members of the rhodopsin family of GPCRs occurs in IC2 at an equivalent position to the

GluLysLysTyr motif of the PTH/PTHrP receptor. In the α_{1B}-adrenergic receptor, mutation of the AspArgTyr to AlaArgTyr resulted in a constitutively active receptor. Mutation of the Arg^{143} residue however caused a reduction in inositol phosphate production. The authors concluded that the Arg^{143} residue mediates receptor activation by allowing several residues in IC2 and IC3 to interact (Scheer et al., 1996). In fact all three intracellular loops have been implicated in PLC activation by the glycoprotein hormone receptors, the adrenergic and the muscarinic receptors (O'Dowd et al., 1988; Chazenbalk et al., 1990; Dohlman et al., 1991; Savarese et al., 1992; Strader et al., 1994). Kosugi et al. (1992) identified a residue in IC3 of the TSH receptor, Ala^{623}, which was required for inositol phosphate production. Mutation to Lys or Glu resulted in impaired IP signalling without affecting cAMP production. However mutation of the equivalent Ala to any other residue in the α_{1B}-adrenergic receptor resulted in a receptor constitutively active for inositol phosphate production (Kjelsberg et al., 1992).

ii) Interactions between transmembrane domains

The model of GPCR structure suggested by Baldwin (1993;1994) based on the low resolution structure of bovine rhodopsin (Schertler et al., 1993) and analysis of over 200 GPCRs has many features confirmed by biochemical analysis. Studies of the PTH/PTHrP receptor structure by Gardella et al. (1996) are entirely consistent with an interaction between TM2 and TM7. Mutation of Gln^{451} in TM7 inhibited PTH(1-34) binding as did mutation of Arg^{230} in TM2. However the double mutation restored binding but prevented any PTH(1-34) stimulation of cAMP or [^{3}H]IP production. Binding of the antagonist/partial agonist PTH(3-34) was unaffected by the mutations. The activation domain of PTH is in its N-terminus and other evidence shows that this region of the peptide binds to residues in EC3. Lee et al. (1995) prevented PTH binding by deleting residues 431-440 in

EC3. Trp^{437} and Gln^{440} were identified as important for binding the N-terminus of PTH. The Arg^{230} residue in TM2 of the PTH/PTHrP receptor is one of three residues identified by Turner et al. (1996) as being important for signalling (Ser^{227}, Arg^{230} and Ser^{233}) after adopting a strategy of mutating charged residues. These residues are thought to form part of a polar face of this transmembrane domain which participates in interhelical ionic interactions which stabilise the active or inactive conformations of this receptor. A study of rhodopsin receptor structure revealed a salt bridge between TM7 (Lys^{296}) and TM3 (Glu^{113}) disruption of which produced a constitutively active receptor (Robinson et al., 1992; Rao et al., 1994). Kudo et al. (1996) used FSH and LH receptor chimaerics to define an interaction between TM5 and TM6 of the LH receptor which is critical for keeping the receptor in an inactive state. Hwa et al. (1996) showed that constitutively active mutants of the α_{1A}- and α_{1B}-adrenergic receptors which had a single point mutation in either TM5 or TM6 could be silenced by a complementary mutation in TM6 or TM5 respectively.

Receptor activation by ligand and transduction of the extracellular signal to the cell interior is thought to occur as a result of small changes in the receptor's conformation induced by ligand binding to several domains in its extracellular surface (for the muscarinic and adrenergic receptors however, the ligand binding site is in the receptor's body (Dixon et al., 1987; Rubenstein et al., 1987; Wess, 1993) as is the site for activation of the rhodopsin receptor (Zhukovsky et al., 1991)). From studies carried out on the rhodopsin receptor (Farahbakhsh et al., 1993; Farahbakhsh et al., 1995) activation is believed to involve a small outward movement of TM3, a small outward movement of the C-terminal end of TM6 accompanied by a clockwise rotation of approximately 30^{o} and a small structural change in IC2. Cross-linking of the cytoplasmic ends of TM3 and TM6 has been shown to prevent activation (Farrens et al., 1996). The exchange site for creation of

the TM3 chimaerics occurs in a region of the receptor which has been demonstrated as being crucial for the function of related receptors. It is therefore possible that this exchange disrupts the precise arrangement of transmembrane domains and intracellular loops required for G protein activation or makes a crucial residue inaccessible thereby disrupting signalling.

4.4.2 C-terminal Truncations

The $VIP_2\Delta$(1-376) truncated receptor

Expression of the $VIP_2\Delta$(1-376) truncated receptor was not detected at the cell surface in whole cell ligand-binding experiments and proved unable to mediate cAMP production in response to ligand. The truncation point for this construct is at the C-terminal end of TM7 and is several residues before the conserved GluValGln motif. Huang et al. (1995b) found that a truncated PTH/PTHrP receptor which was truncated immediately before the GluValGln motif was not successfully expressed at the plasma membrane either but was found localised around the nucleus of the cell. The presence of the GluValGln motif itself however does not seem to be the crucial factor since its mutation to AlaAlaAla had little effect on expression levels or location. It may be the intrusion of the truncation point into the transmembrane region that affects the expression of the receptor by gross structural disruption. Schneider et al. (1994) were unable to express a human PTH/PTHrP receptor truncated before the Val of the GluValGln motif. Findlay et al. (1994) successfully expressed a calcitonin receptor truncated before the Gln of this motif. Internalisation of the receptor was inhibited slightly and sCT-induced cAMP and Ca^{2+} signalling were impaired with respect to the wild-type receptor. Unson et al. (1995) attempted to express truncated rat glucagon receptors and found that receptors truncated in the mid-region of IC1, IC2 and IC3 were not expressed at the plasma membrane of the cell (as determined by

immunofluorescence in permeabilised cells). Binding studies on membranes prepared from cells transfected with the constructs revealed no detectable binding. The authors concluded that all 7 TM regions were required for correct processing of the receptor and its transport to the plasma membrane. The data for such a small group of constructs cannot strongly support such a conclusion. Holtmann et al. (1996), as previously described, successfully expressed truncated VIP_1 and secretin receptors which consisted of the N-terminus, TM1 and a portion of IC1 . Cao et al. (1995) also successfully expressed a similar portion of the rat PACAP receptor demonstrating that all seven TM regions are not required. Holtmann et al. (1996) and Cao et al., (1995) used radioligand binding studies carried out on membrane preparations from transfected cells to determine whether the constructs were being successfully expressed.

The truncated $VIP_2\Delta(1\text{-}391)$ receptor

The 391 amino acid truncated receptor, $VIP_2\Delta(1\text{-}391)$, has a C-terminal tail of 13 amino acids. It was expressed at a similar level to the $VIP_{2\ wt}$ receptor and displayed a similar affinity for PACAP-27 binding. One of the more surprising effects that has been observed for C-terminal truncations in this family of receptors is an increase in ligand-binding affinity. Findlay et al. (1994) found that C-terminal truncation of the porcine calcitonin receptor caused an increase in its affinity for sCT. The binding of sCT occurs in an irreversible manner which is not true of pCT. The truncations were expressed at varying levels in stably transfected HEK 293 cells in a range from 10 million receptors per cell for the wild-type receptor to 52,000 per cell for the lowest expressed truncation. Transformations of the data reveal a clear linear relationship between the log Kd and log receptor number. The authors claim that the increased Kd was not an artefact of the increased receptor number because a similar relationship between construct and ligand-binding affinity

was observed when the receptors were transiently expressed in COS 1 cells at a uniform level. The only K_d values quoted by the authors however are those for the stable transfectants. It is assumed by the authors that the C-tail assists in maintaining the receptor in a low-affinity state as described by Lefkowitz et al. (1993) and Samama et al. (1993). Unson et al. (1995) described a slight increase in ligand binding affinity for glucagon receptors truncated to 443 and 415 amino acids from the full-length 483. Parker and Ross (1991) provided an interesting example of the effect of C-terminal truncation in their study on the avian β-adrenergic receptor. For the truncated receptor they observed increased basal and agonist-stimulated adenylate cyclase stimulation, agonist activity for classical antagonists at the receptor and an increase in ligand-binding affinity for agonist. In this case the C-terminus appears to regulate coupling efficiency to adenylate cyclase as well as modulating the receptor's affinity for agonist. This data is supportive of the idea that the C-terminus can constrain or stabilise the receptor in an inactive conformation. It is now believed that GPCRs can adopt a range of different conformations (Kenakin, 1995a; Kenakin 1995b) in the presence of agonists, antagonists, inverse agonists etc. If the role of the antagonist is to stabilise the receptor in the inactive conformation, then the absence of the C-terminus, which has also been credited with this role, may mean that the receptor has a much higher probability of being in an active conformation when encountered by the antagonist. An alternative possibility is that the antagonist induces a conformational change which is distinct from the resting/inactive state but is energetically unfavourable in the absence of the C-terminus and is therefore readily converted to an active conformation.

The role of the C-terminal tail in the ability of the human PTH/PTHrP receptor to stimulate adenylate cyclase has been elucidated in experiments by Schneider et al. (1994) who produced a series of progressively C-terminally

truncated PTH/PTHrP receptor constructs. A receptor with a C-terminal tail of 19 amino acids was functionally expressed in COS 1 cells without being noticeably different from the wild-type receptor in its ability to stimulate adenylate cyclase or PLC or in its ligand binding affinity for [Tyr^{36}]cPTHrP-(1-36). Nevertheless when the wild-type receptor was expressed in HEK293 cells it was unable to mediate any stimulation of PLC, despite there being 155±44 ($x10^3$) binding sites per cell. The authors estimate this as being 10% of the expression level in COS 1 cells. In HEK 293 cells the wild-type receptor had an EC_{50} for cAMP production identical to that displayed by the receptor in COS 1 cells (9.41±0.09 and 9.30±0.10 (-log(M) respectively for hPTH-(1-38)). The authors found that truncating the C-terminus to a point between residues 504 and 483 restored the ability of the receptor to stimulate PLC. The receptor body is therefore thought to interact promiscuously with G proteins while the C-terminus serves to restrict this interaction with some G proteins more than others, thus in this case directing the receptor towards an interaction with G_s.

The potency of VIP for cAMP-production mediated by $VIP_{2\ wt}$ and $VIP_2\Delta$(1-391) receptors was very similar when expressed in COS 7 cells as were the maximum stimulations and basal levels of cAMP production. It is interesting to note that when expressed as stable transfectants in CHO cells the rat VIP_1 and VIP_2 receptors proved unable to mediate significant stimulation of PLC while AC stimulation appeared unaffected ([^{3}H]IP production of 0.92±0.06 - and 1.03±0.07-fold of basal control for VIP_1 and VIP_2 receptors respectively after a 60 minute stimulation with 3 μM VIP). In HEK293 cells however, the human VIP_2 receptor mediated a stimulation of PLC with a maximum response 10-fold greater than that observed in COS 7 cells (data not shown). The effects of C-terminal truncation have not been examined on the responses in cell lines other than COS 7 cells. The characteristics of the responses observed in the COS 7 cell transient expression

system closely resemble the responses mediated by the endogenous VIP_2 receptor in GH_3 cells, a rat pituitary tumour cell-line (see Chapter 5).

Neither concentration-response curves nor timecourses revealed any differences between [^{3}H]IP production mediated by the $VIP_2\Delta(1\text{-}391)$ receptor and that mediated by the $VIP_{2\ wt}$ receptor. In contrast to a number of older studies on related receptors, the wild-type and truncated receptors appear to interact with the same G proteins and with similar efficiency. It must be kept in mind that the $VIP_2\Delta(1\text{-}391)$ construct still has a C-terminal tail of 13 amino acids which includes the whole of the predicted helical region and may be sufficient for a number of C-terminal tail-regulated activities. Huang et al. (1995; 1995b) carried out a study on the effects of C-terminal truncation on the signalling and expression of the opossum PTH/PTHrP receptor. A mutant with a C-terminus of 9 amino acids was expressed and functioned normally with respect to adenylate cyclase stimulation, Ca^{2+} signalling (as measured in *Xenopus* oocytes by monitoring ^{45}Ca efflux from prelabelled pools) and ligand binding. The truncated receptors were however expressed at a lower level than the wild-type. This lower level of expression did not affect second-messenger production presumably because there was still an excess of expressed receptor available for this task. The authors make the point that Iida-Klein et al. (1995), working at a much lower expression level, observed enhanced AC stimulation as a result of C-terminal truncation of the rat PTH/PTHrP receptor, such an effect might be missed if the receptor number was not a limiting factor.

Endocytosis and Desensitisation

Huang et al. (1995b) did however identify a significant effect of the truncations on the internalisation of the PTH/PTHrP receptor. Mutation of the GluValGln motif to AlaAlaAla resulted in a 40% increase in ligand endocytosis.

The authors interpreted this as being due to the loss of a signal that negatively regulates endocytosis. Application of a hypertonic sucrose solution caused an 80% reduction in internalisation which is attributed to the disruption of clathrin lattices involved in coated pit formation (Hansen et al., 1993). Silve et al. (1982) had already demonstrated the accumulation of iodinated PTH in clathrin-coated pits on the surface of cells in clavarial bone. The deletion of residues 475-494 in the C-terminal tail caused a 50-60% decrease in ligand internalisation suggesting that this constitutes a positive signal for endocytosis. It is possible therefore that the C-terminal tail of the opossum(OK) PTH/PTHrP receptor contains both inhibitory and facilitatory domains for endocytosis.

Reneke et al. (1988) found that truncation of the yeast α-factor receptor C-terminus caused a significant reduction in ligand-induced endocytosis. Nussenzveig et al. (1993) determined that there were two domains in the TRH receptor C-terminus that facilitate fast TRH-induced endocytosis. There are a number of examples however of C-terminal deletions increasing or facilitating internalisation as observed by Huang et al. (1995b) with the GluValGln to AlaAlaAla mutation in the PTH/PTHrP receptor. The LH/CG receptor C-terminus appears to act to reduce the rate of internalisation of the receptor (Rodriguez et al., 1992). The majority of mammalian forms of adrenergic receptor undergo agonist-induced desensitisation whereas the avian form does not. Hertel et al. (1990) found that the removal of residues from the avian β_1-adrenergic receptor C-terminus allowed ligand-induced endocytosis to occur. The mechanism for regulation of this ligand-induced endocytosis is not known although Urena et al. (1994) have observed that both receptor availability and cAMP accumulation are controlled by certain steroids and cytokines. Blind et al. (1993) identified the C-terminal tail of the rat PTH/PTHrP receptor as a substrate for phosphorylation by PKC, PKA and βARK1 *in vitro*. Blind et al. (1995) demonstrated the

constitutive phosphorylation of the OK PTH/PTHrP receptor stably expressed in HEK293 cells. The authors found that occupancy of the receptor by activating hormone increased phosphorylation. Phosphorylation of the receptor could be stimulated by the phorbol ester PKC activator, phorbol 12-myristate 13-acetate (PMA) and the stimulator of cAMP production and PKA activation, forskolin. The agonist-induced phosphorylation however could not be blocked by the PKC inhibitor, GF 109203X, the PKA inhibitor, H-89 or the broad-spectrum kinase inhibitor, staurosporine which inhibits both kinases at high dose. A role for PKC in this system is not strongly supported since the wild-type human PTH/PTHrP receptor (Schneider et al., 1994) and the OK PTH/PTHrP receptor (Blind et al., 1995) expressed in HEK 293 cells were unable to stimulate PLC. The authors concluded that a non-second messenger-activated kinase such as a G protein-coupled receptor kinase (GRK) is probably involved. The β-adrenergic receptor kinase (βARK) can mediate the homologous desensitisation of the AC response stimulated by β-adrenergic receptor agonists (Hausdorff et al., 1990) and these related GRK family kinases (for review see: Premont et al., 1995) are likely candidates for mediating desensitisation of the VIP_2 receptor also.

Staurosporine has a potentiating effect on agonist-stimulated [^{3}H]IP production mediated by the $VIP_{2\ wt}$ and $VIP_2\Delta(1\text{-}391)$ receptors but also has a potentiating effect on $AlCl_3$/NaF stimulated [^{3}H]IP production indicating that the effect is dependent on G protein activation but is downstream of the receptor. The C-terminal portion of the receptor contains four putative PKC phosphorylation sites conforming to the PKC consensus specificity motifs as defined by Pearson & Kemp (1991). Phosphorylation of the receptor itself has of course not been demonstrated in this experiment and the action of PKC need not be directly on the receptor. If the receptor were phosphorylated it may not be on residues in the C-terminal tail. The phosphorylation may occur at a residue in the receptors body

exposed as a result of an interaction between the C-terminal tail and the intracellular portion of the receptor's body. The $VIP2_{wt}$ receptor-mediated IP response was unaffected by treatment with the PKC inhibitor GF 109203X (data not shown) indicating a lack of tonic influence under the conditions assessed. Turner et al. (1988) demonstrated that PMA caused a decrease in VIP-stimulated AC activity and in cell-surface VIP receptors in the HT29 human colonic adenocarcinoma cell line but concluded that PKC was not involved in agonist-induced desensitisation. It should however be considered that PKC activation mediated by another receptor could be relevant to modulation of VIP receptor function *in vivo*.

4.4.3 Summary

In summary, the IC3 region of the $VIP2_{wt}$ receptor has been identified as the major functional domain involved in the activation of a PTx-sensitive G protein by which the receptor mediates PLC stimulation. The N-termini of the $VIP2_{wt}$ and PACAP receptors have been identified as the major determinants of ligand-binding specificity by virtue of their effect on the recognition of VIP as opposed to PACAP-38 by each receptor. Agonists for these receptors appear to undergo 'two-site binding', with auxiliary sites in the receptor body being required in addition to the N-terminus for wild-type receptor responses. The PACAP receptor appears to contain elements which improve its expression level as well as having a C-terminus which facilitates an improved interaction with G_s when inserted into the $VIP2$ receptor. However the whole of the PACAP receptor body appears to be required for optimal stimulation of AC. The presence of the $VIP2_{wt}$ receptor C-terminus conversely appears to have a negative influence on $G_{q/11}$ stimulation by the PACAP receptor. The TM3 region of the receptor seems likely to be critical for receptor function since its disruption in the V3P chimaerics was the probable cause

of attenuated cAMP and IP signalling. Many interactions between transmembrane regions and intracellular loops have been postulated (for review see Wess, 1997) and it is not possible at this stage to determine which of these may have been affected. The removal of the $VIP_{2\ wt}$ receptor C-terminus had no significant effects on the cAMP or IP signalling. PKC phosphorylation may have a role to play in modulating the internalisation of this receptor, a phenomenon which has not been investigated in this study.

In studies such as this, the receptor-mediated signalling phenomena should ideally be observed at low expression levels since this is a more sensitive way of examining effects on tightly-coupled G protein mediated signalling pathways. Consideration should also be given to the difference in coupling efficiencies between pathways activated by a single receptor. Many studies claim to demonstrate selective disruption of a signalling pathway but this is inevitably the less tightly-coupled response and close examination of the data often reveals a decrease in the activity of the other response also. In this family of receptors, any structural manipulation which lead to the disruption of AC stimulation without affecting PLC stimulation would be a more convincing demonstration of separable domains for coupling to different G proteins than disruption of PLC stimulation.

Table 4.1 Relative expression levels, IC_{50} values for homologous displacement of [^{125}I]PACAP-27, EC_{50} and maximal stimulation values (fold of basal control) for PACAP-38 and VIP-stimulated cAMP and [^{3}H]inositol phosphate production mediated by wild-type and chimaeric receptors transiently expressed in COS 7 cells.

Receptor	Specific [^{125}I] PACAP-27 Binding		cAMP Production				[^{3}H]IP Production			
			PACAP-38		VIP		PACAP-38		VIP	
	B_{max} (fmol/ 10^5 cells)	IC_{50} (nM)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)
$PACAP_{wt\ long}$	520±52	31±3	0.5±0.4	22.4±1.1	38.6±3.8	12.08±0.5	17.6±0.6	11.1±0.3		nd
$PACAP_{wt\ short}$	494±37	30±1	0.7±0.1	19.9±1.4	28.6±2.0	15.7±0.2	10.6±0.6	9.8±0.7		nd
$P_{long}7V$	306±27	24±2	1.4±0.1	20.1±0.9	81.7±0.1	12.6±0.4	16.3±0.6	5.95±0.2		nd
$P_{short}7V$	228±17	16±1	0.6±0.1	18.4±0.5	42.9±2.4	16.7±0.6	32.0±2.0	4.19±0.3		nd
P_5V	202±39	30±6	0.3±0.01	10.5±0.5	80.1±19.3	4.3±0.5	48.0±2.9	3.2±0.01		-
P_3V	234±57	69±17	0.7±0.1	10.1±0.5	19.0±1.3	7.5±0.2	43.2±7.0	1.6±0.1		-
P_1V	130±32	70±17	0.6±0.2	12.7±0.3	302.5±24.8	8.5±0.2	55.6±22.9	1.3±0.1		nd
$VIP_{2\ wt}$	59±5	19±1	0.9±0.1	7.8±0.5	0.3±0.1	8.2±0.1	36.9±8.1	3.0±0.3	45.0±6.6	2.4±0.1
V_7P	46±15	16±5	0.4±0.1	12.5±0.5	0.1±0.02	13.4±0.3	40.6±3.6	1.8±0.1		-

	Receptor	Specific [^{125}I] PACAP-27 Binding		cAMP Production PACAP-38		VIP		[^{3}H]IP Production PACAP-38		VIP	
		B_{max} (fmol/ 10^5 cells)	IC_{50} (nM)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)	EC_{50} (nM)	E_{max} (fold of basal control)
	V_5P_{long}	228±20	58±4	0.6±0.1	13.1±0.5	0.4±0.1	12.81±0.6	59.3±16.5	1.6±0.1		-
	V_5P_{short}	163±21	38±4	0.8±0.1	12.4±0.6	0.6±0.1	11.36±0.8	63.6±10.4	1.7±0.0		-
	V_3P_{long}	260±36	72±10	0.2±0.02	6.3±0.1	2.3±0.1	4.2±0.3		nd		nd
	V_3P_{short}	91±24	56±14	0.5±0.1	7.0±0.2	3.4±0.4	4.7±0.2		nd		nd
	V_1P_{long}	150±14	13±1	0.6±0.2	30.1±1.4	0.7±0.1	33.1±4.6	15.5±1.3	9.4±0.4	31.3±0.6	10.3±0.0
	V_1P_{short}	215±22	16±2	0.6±0.3	27.3±1.7	0.4±0.1	32.4±1.1	15.7±2.8	7.2±0.3	20.3±1.3	8.6±0.0

Bmax and IC_{50} values were determined by non-linear curve fitting of data from homologous displacement of ligand-binding experiments carried out on whole cells at 0°C for 60 minutes. EC_{50} values for [^{3}H]IP production were derived from non-linear curve fitting of concentration response curves. Values are expressed as means±SEM (n=3-6). Bmax is expressed as fmol/ protein equivalent of 10^5 cells. EC_{50} is expressed in nM. The maximal stimulation (E_{max}) is expressed as 'fold of basal control'. A typical basal value for cAMP production was 6±2 pmol/ well. A typical basal value for [^{3}H]inositol phosphate production was 7700±600 dpm per well. EC_{50}s, IC_{50}s and E_{max} values were derived from the non-linear curve-fitting programme P-fit (Elsevier Biosoft, Cambridge, UK). 'nd' indicates that no stimulation was detected at agonist concentrations up to 3 μM.

Fig. 4.1 Homologous displacement of [^{125}I]VIP binding to COS 7 cells expressing the $VIP_{2\ wt}$ and $VIP_2\Delta$(1-391) receptors.

The binding assay was performed at 0^{o}C for 60 minutes on whole COS 7 cells transiently expressing the$VIP_{2\ wt}$ (□) or $VIP_2\Delta$(1-391) (○) receptors. The values are the means±SEM, n=8. The $VIP_{2\ wt}$ and $VIP_2\Delta$(1-391) receptors had IC_{50} values of 7.0±3.7 and 6.0±1.1 nM respectively and B_{max} values of 0.19±0.04 and 0.11±0.04 pmol/mg protein respectively. [^{125}I]VIP was present at a concentration of 6.15±0.3 pM.

Fig. 4.2 Concentration response curves for VIP-evoked cAMP production mediated by the $VIP_{2\ wt}$, $VIP_2\Delta$(1-391) and $VIP_2\Delta$(1-376) receptor constructs.

The assay was performed on COS 7 cells transiently expressing the $VIP_{2\ wt}$(□), $VIP_2\Delta$(1-391) (○) or $VIP_2\Delta$(1-376) (◇) receptors. Stimulation was for 15 minutes in the presence of 0.5 mM IBMX. Accumulated total cAMP was measured. Basal levels were 7±1 pmol/well. n=4.

Figure 4.1

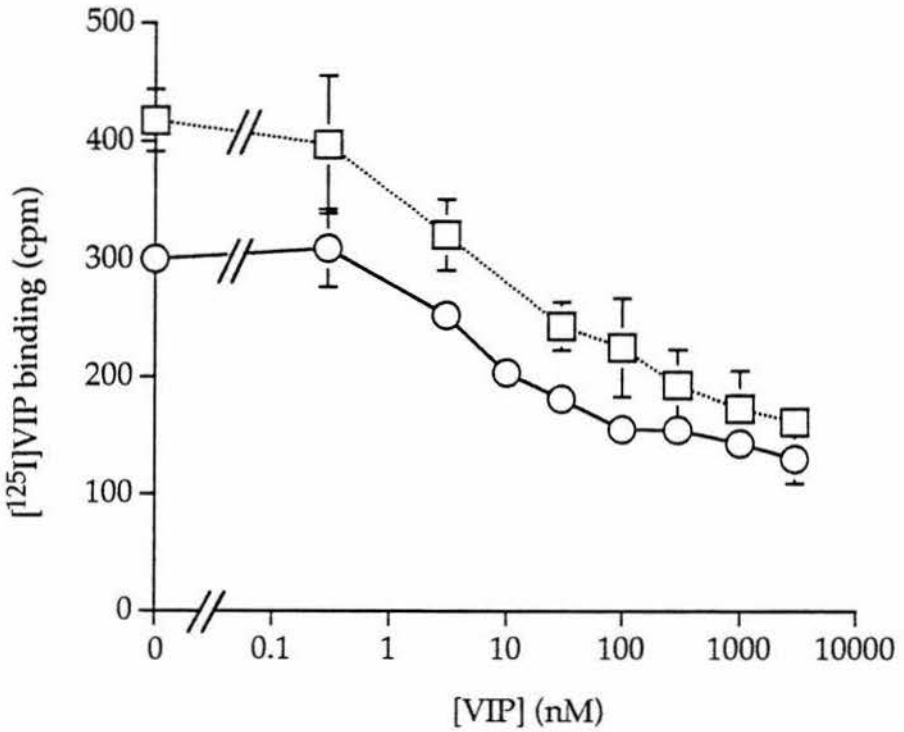


Figure 4.2

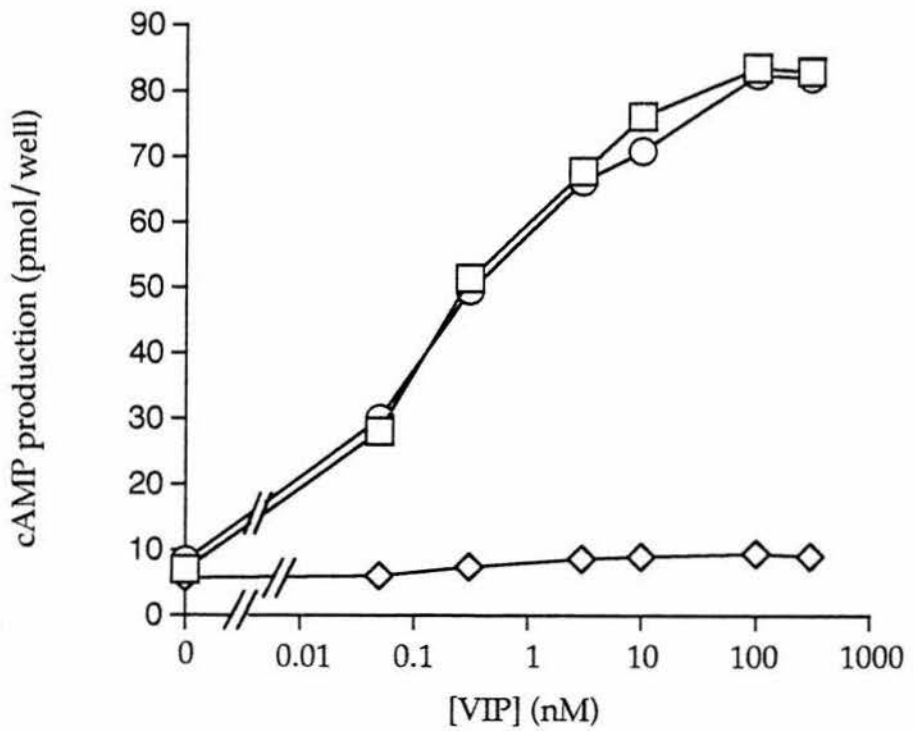


Fig. 4.3 The time-dependence of VIP-evoked cAMP production mediated by the $VIP_{2\ wt}$ and $VIP_2\Delta(1\text{-}391)$ receptors

a) Intracellular cAMP accumulation mediated by the $VIP_{2\ wt}$ (□) and $VIP_2\Delta(1\text{-}391)$ (○) receptors. Basal levels were 5±1 pmol/well. b) Total cAMP accumulation. basal levels were 6±2 pmol/well. c) The averaged rate of cAMP accumulation for each time point. The receptors were transiently expressed in COS 7 cells. cAMP accumulation was measured in the presence of 0.5 mM IBMX. Values are means±SEM. n=3.

Figure 4.3

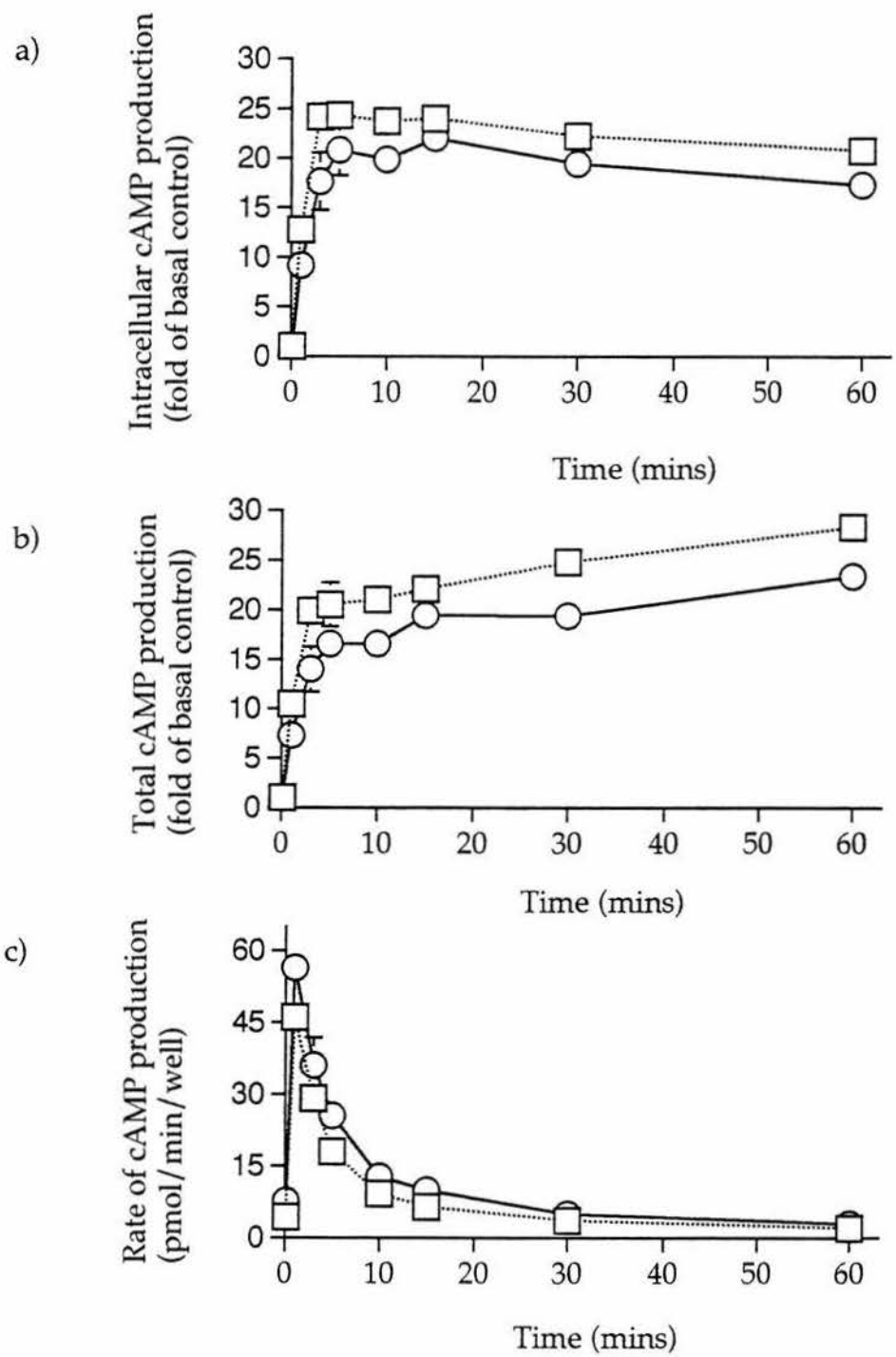

a)
Intracellular cAMP production (fold of basal control)
30
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (mins)
b)
Total cAMP production (fold of basal control)
30
25
20
15
10
5
0
0
10
20
30
40
50
60
Time (mins)
c)
Rate of cAMP production (pmol/min/well)
60
45
30
15
0
0
10
20
30
40
50
60
Time (mins)

Fig. 4.4 Concentration-response curve for VIP-evoked [^{3}H]inositol phosphate production mediated by the $VIP_2\Delta(1\text{-}391)$ receptor.

The receptor was transiently expressed in COS 7 cells and stimulated with the indicated concentrations of VIP for 60 minutes in the presence of 10 mM LiCl. Basal values were 10,334±317 dpm per well. The data are means±SEM from a typical experiment. n=3.

Fig. 4.5 Concentration-response curve for PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the $VIP_2\Delta(1\text{-}391)$ receptor

The receptor was transiently expressed in COS 7 cells and stimulated with the indicated concentrations of PACAP-38 for 60 minutes in the presence of 10 mM LiCl. A typical basal value was 11,362±434 dpm per well. The data are means±SEM from a typical experiment. n=3.

Figure 4.4

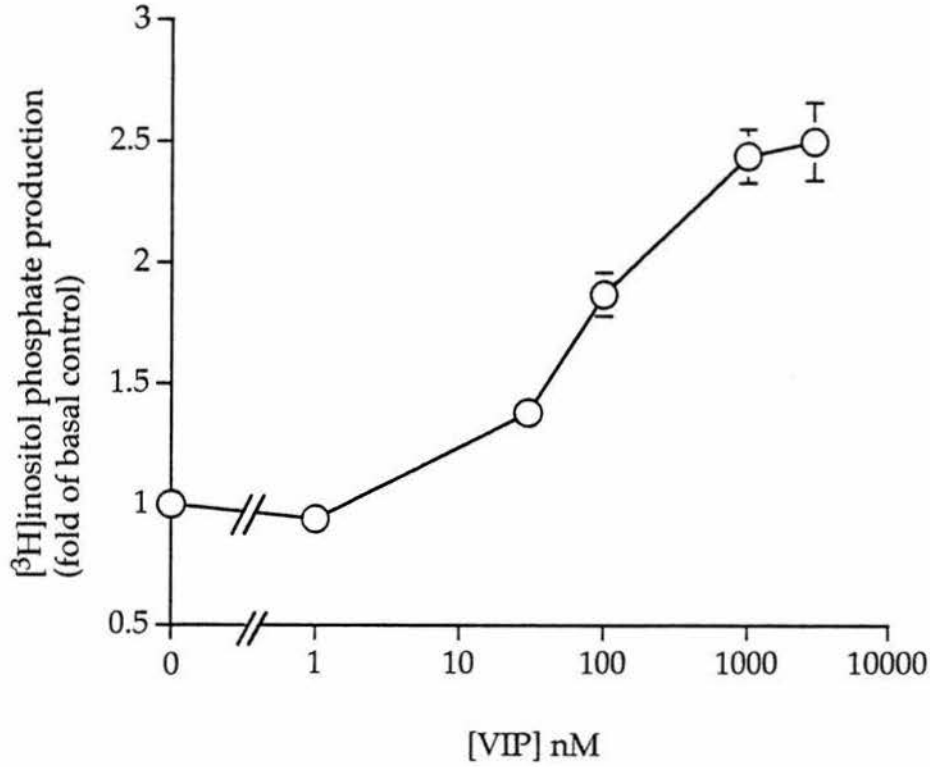


Figure 4.5

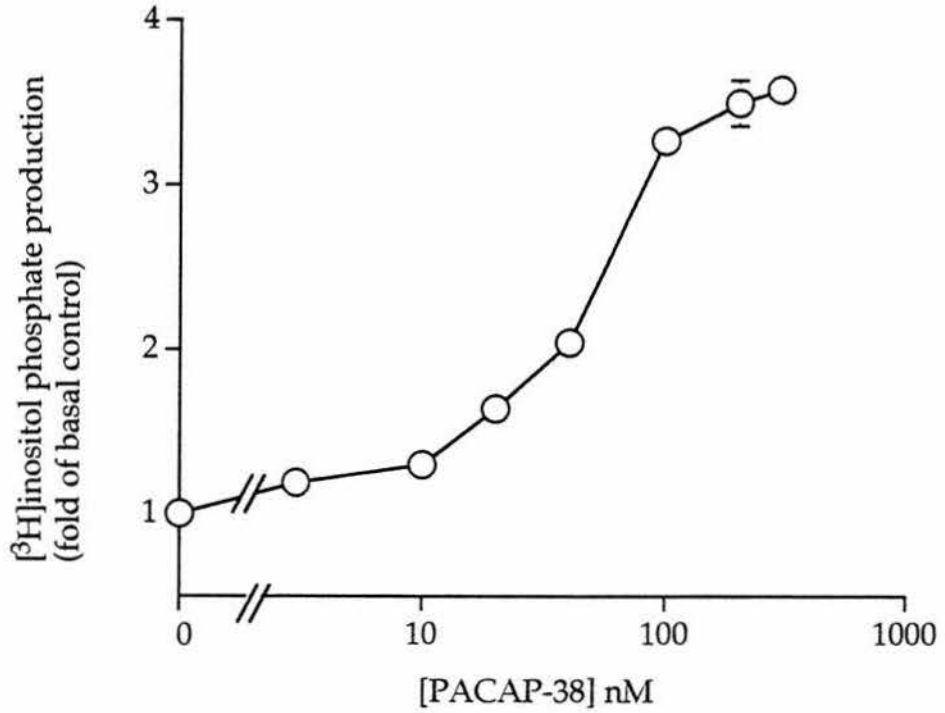


Fig. 4.6 Time course of VIP-evoked [^{3}H] inositol phosphate production mediated by the the $VIP_{2\ wt}$ (□) and $VIP_2\Delta(1\text{-}391)$ (○) truncated receptors.

VIP was added at a concentration of 1 μM. The illustrated data are means±SEM from a typical experiment. There was no significant difference in basal values for cells expressing these receptors. A typical mean basal value was 11,000±200 dpm per well. n=3.

Fig.4.7 Pertussis toxin-sensitive stimulation of PLC mediated by the $VIP_{2\ wt}$ and $VIP_2\Delta(1\text{-}391)$ receptors.

There was no difference in basal [^{3}H]inositol phosphate formation in $VIP_{2\ wt}$ or $VIP_2\Delta(1\text{-}391)$ receptor-expressing cells and data for these is expressed in a combined form. A typical basal activity was 10,314 (±280) dpm/assay. Values are means ± S.E.M.from 6 separate determinations. (*) represents statistically significant inhibition of VIP-induced [^{3}H]inositol phosphate production ($p<0.05$ by Mann-Whitney *U*-test).

Figure 4.6

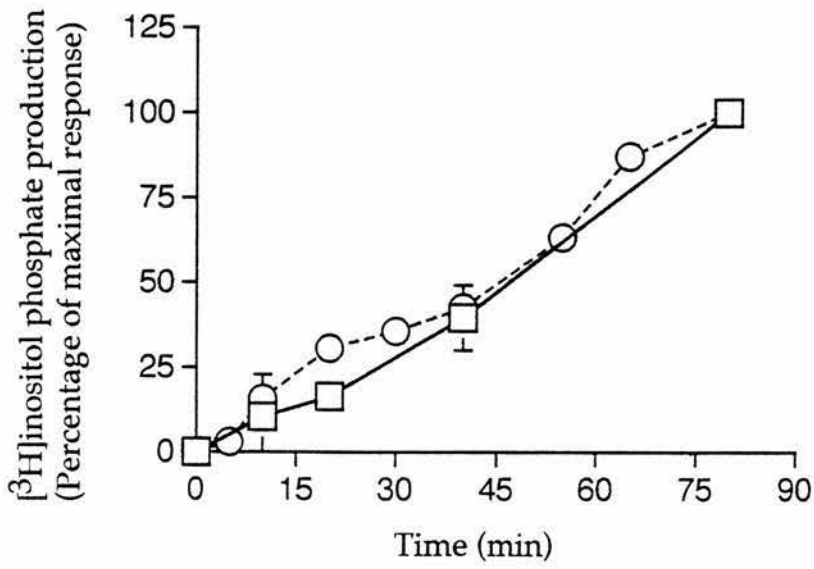


Figure 4.7

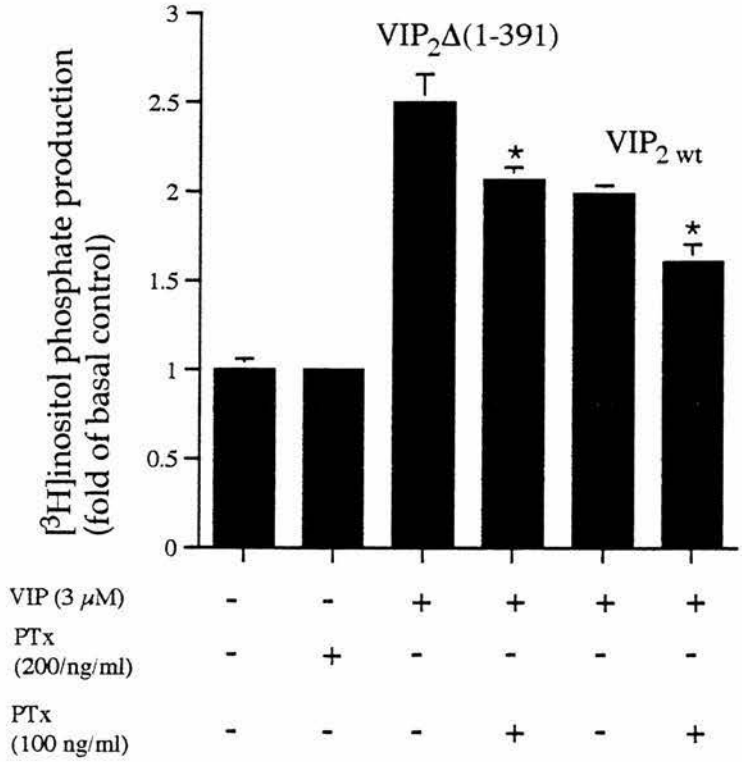


Fig. 4.8 The effects of staurosporine on VIP-evoked [^{3}H]IP production mediated by the $VIP_{2\ wt}$ and $VIP_2\Delta(1\text{-}391)$ receptors.

The $VIP_{2\ wt}$ (open bars) and $VIP_2\Delta(1\text{-}391)$ (filled bars) receptors were transiently expressed in COS 7 cells. Staurosporine (1 μM) was applied for the period of the assay (60 minutes). Staurosporine had no significant effect on basal levels. A typical basal value was 8,187±485 dpm per well. n=6. (*) represents a statistically significant potentiation of VIP-evoked [^{3}H]inositol phosphate production by staurosporine ($p<0.05$ by Mann-Whitney *U*-test).

Figure 4.8

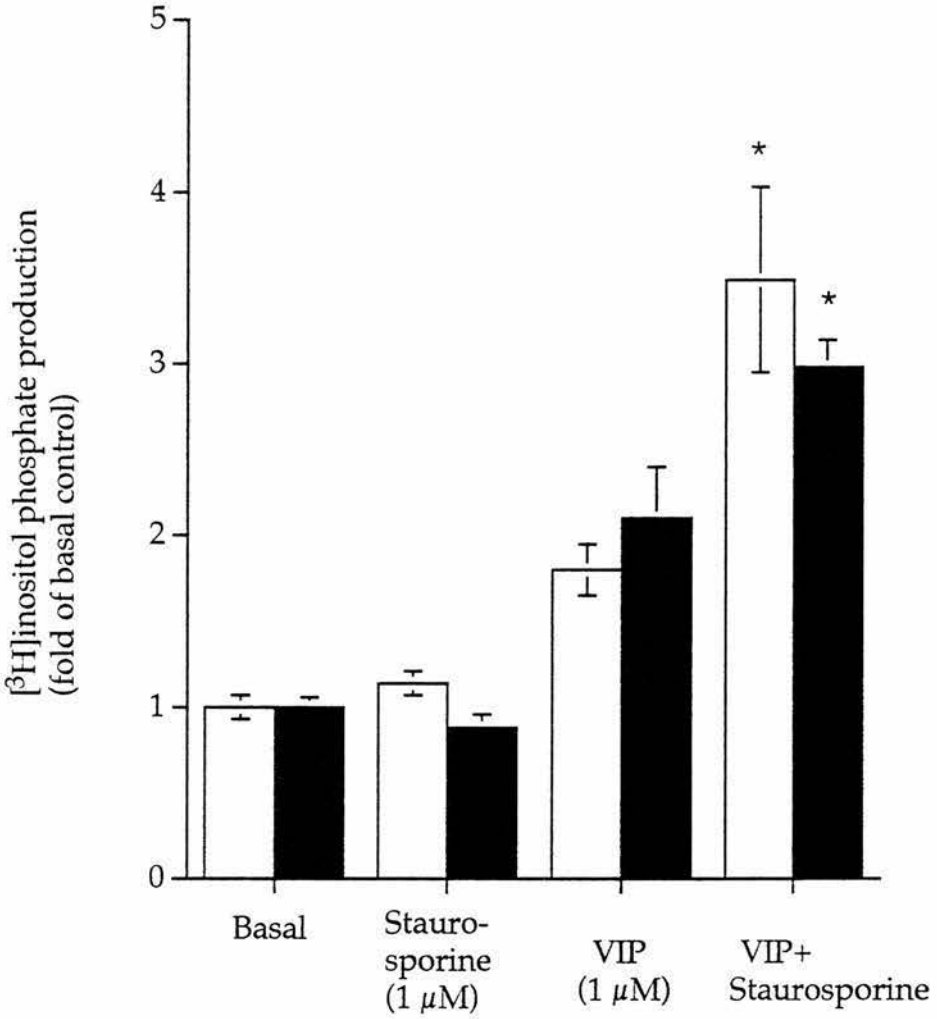
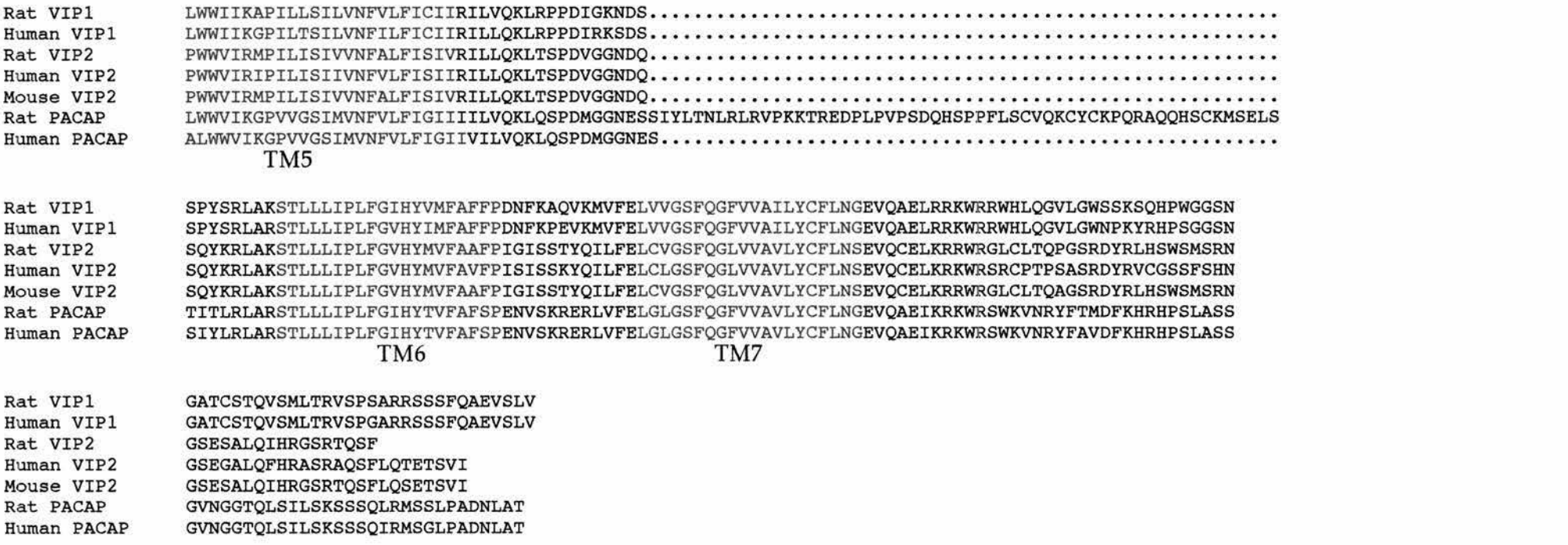

[3H]inositol phosphate production
(fold of basal control)
5
4
3
2
1
0
*
*
Basal
Staurosporine (1 μM)
VIP (1 μM)
VIP+ Staurosporine

Table 4.2 The effect of pertussis toxin pretreatment on PACAP-38 stimulated [^{3}H]inositol phosphate production mediated by wild-type and chimaeric receptors transiently expressed in COS 7 cells.

Receptor constructs	% of control response (±SEM)	n
Wild-type Receptors		
$VIP_{1\ wt}$	55±3*	11
$VIP_{2\ wt}$	59±5*	11
$PACAP_{wt\ long}$	101±3	6
$PACAP_{wt\ short}$	104±3	6
Chimaerics		
P_1V	55±16*	6
P_3V	82±5*	6
P_5V	66±2*	4
V_7P	76±4*	8
V_5P_{long}	108±15	6
V_5P_{short}	113±15	6
$P_{long7}V$	102±6	6
$P_{short7}V$	113±9	6

Cells were treated for 16 hours with 100 ng/ml Pertussis toxin before stimulation with 100 nM PACAP-38 for 60 minutes. The control response is 100 nM PACAP-38 stimulated [^{3}H]inositol phosphate production measured without pertussis toxin pretreatment. (*) Represents statistically significant inhibition of PACAP-38 stimulated [^{3}H]IP production ($p < 0.05$ by Mann-Whitney *U*-test). Values are expressed as means±SEM.



This diagram shows a line-up of the known VIP and PACAP receptor sequences, the residues in the VIP receptors focussed on in various site-directed mutagenesis studies are highlighted.

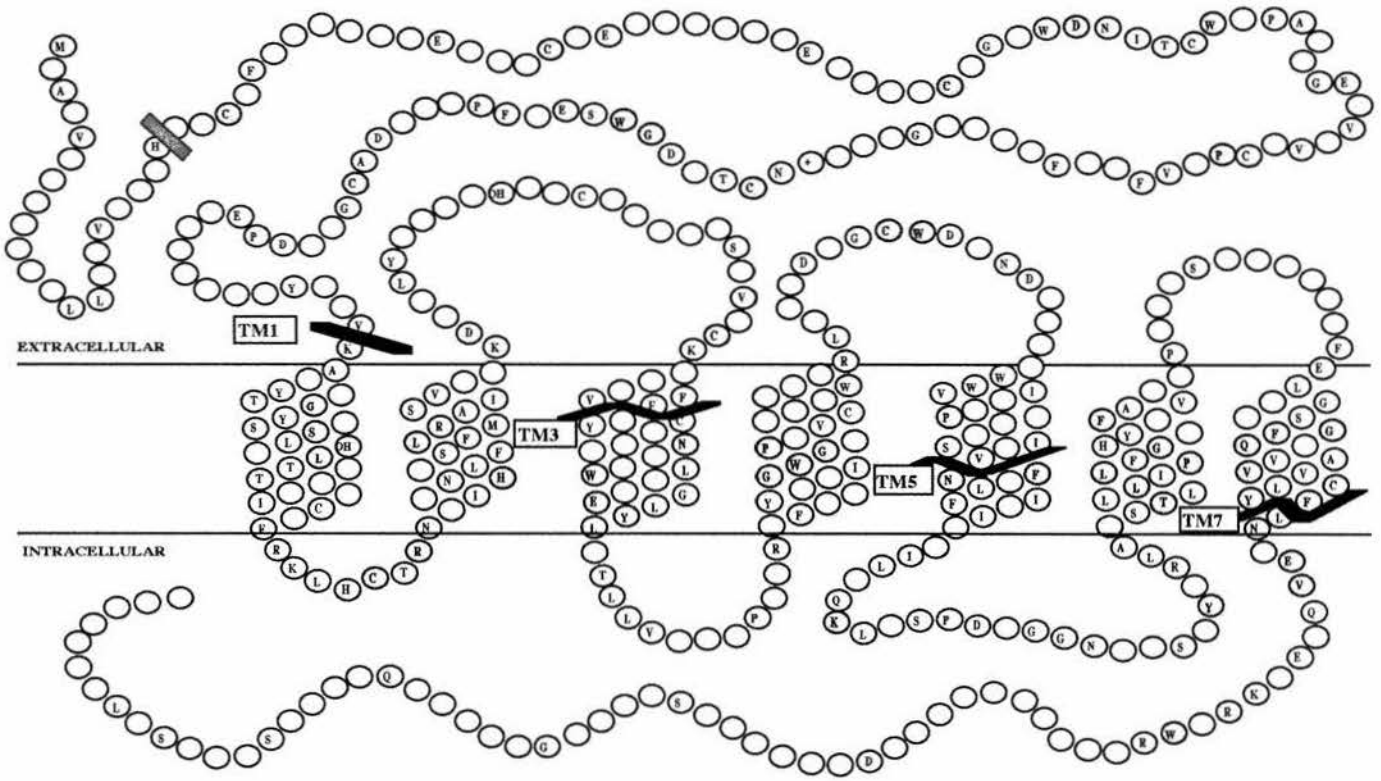
Mutation of the red residues abolished [^{125}I]VIP-binding and VIP-induced cAMP production.
Mutation of the light blue residues reduced [^{125}I]VIP-binding and increased the EC_{50} for VIP-induced cAMP production.
Mutation of the green residues had no effect on [^{125}I]VIP binding.
The underlined Asn residues are glycosylated and important for delivery of the receptor to the plasma membrane
The dark blue residues are in the putative transmembrane regions as determined by a hydrophobicity plot.

(Couvineau et al., 1995; Gaudin et al., 1995; Du et al., 1997; Nicole et al., 1997; Knudsen et al., 1997)

Table 4.3 **Amino acid sequences of the known VIP and PACAP receptors**

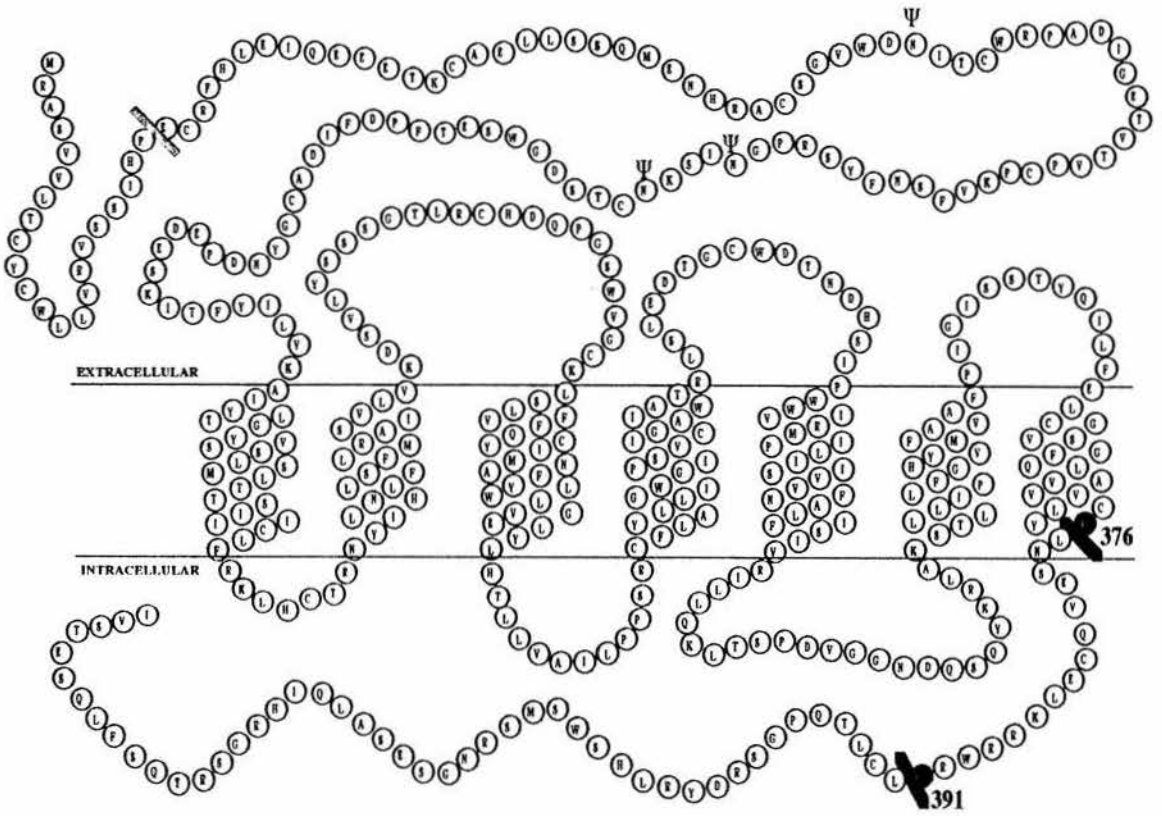
```
Rat VIP1        MRPPSPPHVRWLCVLAGALACALRPAGSQAASPQHECEYLQLIEIQ..RQQCLEEAQLENET.TGCSKMWDNLTCWPTTPRGQAVVLD
Human VIP1      MRPPSPLPARWLCVLAGALAWALGPAGGQAARLQEECDYVQMIEVQ..HKQCLEEAQLENET.IGCSKMWDNLTCWPATPRGQVVVLA
Rat VIP2        ..........MRASVVLTCYCWLLV..RVSSIHPECRFHLEIQE..EETKCAELLSSQMENHRACSGVWDNITCWRPADIGETVTVP
Human VIP2      ..........MRTLLPPALLTCWLLA..QAARLQEECDYVQMIEVQ..HKQCLEEAQLENET.IGCSKMWDNITCWRPANVGETVTVP
Mouse VIP2      ..........MRASVVLTCYCWLLV..RVSSIHPECRFHLEIQE..EETKCAELLSSQTENQRACSGVWDNTCWRPADVGETVTVPI
Rat PACAP       ..........MARVLQLSLTALLLP...VAIAMHSDCIFKKEQAMCLERIQRANDLMGLNESSPGCPGMWDNITCWKPAQVGEMVLVS
Human PACAP     ..........MAGVVHVSLAALLLLP..MAPAMHSDCIFKKEQAMCLEKIQRANELMGFNDSSPGCPGMWDNITCWKPAHVGEMVLVS

Rat VIP1        CPLIFQLFAPIH.....................GYNISRSCTEEGWSQLEPGPYHIACGLNDRASSLDEQQQTKFYNTV
Human VIP1      CPLIFKLFSSIQ.....................GRNVSRSCTDEGWTHLEPGPYPIACGLDDKAASLDEQQ.TMFYGSV
Rat VIP2        CPKVFSNFYSR.....................PGNISKNCTSDGWSETFP.DFIDACGYNDPEDES..KI..TFYILV
Human VIP2      CPKVFSNFYSK.....................AGNISKNCTSDGWSETFP.DFVDACGYSDPEDES..KI..TFYILV
Mouse VIP2      CPKVFSNFYSR.....................PGNISKNCTSDGWSETFP.DFIDACGYNDPEDES..KI..SFYILV
Rat PACAP       CPEVFRIFNPDQVWMTETIGDSGFADSNSLEITDMGVVGRNCTEDGWSEPFP.HYFDACGFDDYEPESGDQD..YYYLSV
Human PACAP     CPELFRIFNPDQVWETETIGESDFGDSNSLDLSDMGVVSRNCTEDGWSEPFP.HYFDACGFDEYESETGDQD..YYYLSV

Rat VIP1        KTGYTIGYSLSLASLLVAMAILSLFRKLHCTRNYIHMHLFMSFILRATAVFIKDMALFNSGEIDHCSEAS...VGCK
Human VIP1      KTGYTIGYGLSLATLLVATAILSLFRKLHCTRNYIHMHLFISFILRAAAVFIKDLALFDSGESDQCSEGS...VGCK
Rat VIP2        KAIYTLGYSVSLMSLTTGSIIICLFRKLHCTRNYIHLNLFLSFMLRAISVLVKDSVLYSSSGTLRCHDQPGSWVGCK
Human VIP2      KAIYTLGYSVSLMSLATGSIILCLFRKLHCTRNYIHLNLFLSFILRAISVLVKDDVLYSSSGTLHPDQPSSWVCGCK
Mouse VIP2      KAIYTLGYSVSLMSLTTGSIIICLFRKLHCTRNYIHLNLFLSFMLRAISVLVKDSVLYSSSGLLRHDQPASWVCGCK
Rat PACAP       KALYTVGYSTSLATLTTAMVILCRFRKLHCTRNFIHMNLFVSFMLRAISVFIKDWILYAEQDSSHCFVST...VECK
Human PACAP     KALYTVGYSTSLVTLTTAMVILCRFRKLHCTRNFIHMNLFVSFMLRAISVFIKDWILYAEQDSNHCFIST...VECK
                          TM1                                TM2

Rat VIP1        AAVVFFQYCVMANFFWLLVEGLYLYTLLAVSFFSERKYFWGYILIGWGVPSVFITIWTVVRIYFEDFGCWDTIINSS
Human VIP1      AAMVFFQYCVMANFFWLLVEGLYLYTLLAVSFFSERKYFWGYILIGWGVPSTFTMVWTIARIHFEDYGCWDTIN.SS
Rat VIP2        LSLVFFQYCIMANFYWLLVEGLYLHTLLVAILPP.SRCFLAYLLIGWGIPSVCIGAWIATRLSLEDTGCWDTNDHSI
Human VIP2      LSLVFLQYCIMANFFWLLVEGLYLHTLL AMLPP.RRCFLAYLLIGWGLPTVCIGAWTAARLYLEDTGCWDTNDHSV
Mouse VIP2      LSLVFFQYCIMANFYWLLVEGLYLHTLLVAILPP.SRCFLAYLLIGWGIPSVCIGAWTATRLSLEDTGCWDTNDHSI
Rat PACAP       AVMVFFHYCVVSNYFWLFIEGLYLFTLLVETFFPERRYFYWYTIIGWGTPTVCVTVWAVLRLYFDDAGCWDMNDSTA
Human PACAP     AVMVFFHYCVVSNYFWLFIEGLYLFTLLVETFFPERRYFYWYTIIGWGTPTVCVTVWATLRLYFDDTGCWDMNDSTA
                          TM3                                TM4
```

Fig. 4.9 Exchange sites used for construction of chimaeric VIP2/PACAP receptors.



The black bars labelled TM1, TM3, TM5 and TM7 indicate the position of the exchange sites used create the TM1, TM3, TM5 and TM7 chimaerics respectively. The residues identified by single letter code are conserved between the VIP2 and PACAP receptors, the grey residues are also conserved in the other members of this receptor family. The grey bar indicates the site at which the signal sequence is cleaved from the N-terminus.

Fig. 4.10 Carboxyl-terminal truncation sites in the rat VIP_2 receptor



The black bars indicate the positions at which the receptor was C-terminally truncated to create the $VIP_2\Delta(1\text{-}391)$ and $VIP_2\Delta(1\text{-}376)$ mutant receptors. The residues identified in black are the carboxyl terminal residues of the truncated receptors. 'Y' indicates the site of a putative N-linked glycosylation site. The grey bar indicates the site at which the signal sequence is cleaved from the N-terminus.

Chapter 5

Characteristics of native VIP_2 receptors in rat tissues

5.1 Introduction

This study utilised the expression of various receptor constructs in a heterologous expression system as a means of investigating the contribution made by specific VIP_2 receptor domains to its signalling characteristics. In order to make such a study meaningful, the signalling characteristics of the wild-type VIP_2 receptor must be understood. Chapters 3 and 4 dealt with wild-type receptor signalling and the role of specific receptor domains. We have established that in a heterologous expression system (transient expression of the receptor in COS 7 cells) the VIP_2 receptor mediated the stimulation of adenylate cyclase and PI hydrolysis, the latter occurring by a partially PTx-sensitive mechanism. The stimulation of PI hydrolysis was found to be partially attributable to calcium release-activated calcium influx ($CRAC_i$) and it is this part of the process that appears to be largely dependent on a PTx-sensitive G protein. The logical progression of this work required the examination of VIP_2 receptor signalling in its native environment to ensure that our findings in expression systems were consistent with its physiological function.

VIP is recognised as having an important role to play in the regulation of pituitary function. VIP-stimulated PRL release has been demonstrated in vivo and in vitro from the pituitary and from clonal cell lines (Vijayan et al., 1979; Kato et al., 1978; Gourdji et al., 1979; Enjalbert et al., 1980; Murlarkey et al., 1981; Onali et al., 1983; Kato et al., 1984). PACAP stimulation of the VIP_2 receptor in GH_3 cells also causes GH release (Murakami et al., 1995). VIP- and PACAP-like immunoreactivity have been found in rat hypothalamic neurons projecting to the external zone of the median eminence (Koves et al., 1991). The hypophysial portal blood in rat contains high levels of VIP, in the range 400-520 pM (13-17 fold greater

than in peripheral plasma: Shimatsu et al., 1981) and PACAP-38 levels (54-107 pM) were found to be 2-4 fold of peripheral levels (Dow et al., 1994).

The GH_3 cell line was therefore chosen for the examination of signalling mediated by the VIP_2 receptor in its native environment. This cell line was derived from a transplantable rat pituitary tumour in the late 1960's (Tashjian et al., 1968). The anterior pituitary has been observed to express a relatively high number of VIP_2 receptors (Usdin et al., 1994; Rawlings et al., 1995). The anterior pituitary contains 5 types of secretory cell; gonadotrophs, somatotrophs, lactotrophs, corticotrophs and thyrotrophs. GH_3 and GH_4C_1 cells secrete growth hormone(GH) and prolactin(PRL) which means that they have characteristics of both lactotrophs and somatotrophs. Pharmacological studies indicated that somatotrophs express a VIP receptor (Deutsch & Sun, 1992; Rawlings et al., 1995; Murakami et al., 1995). GH_3 and GH_4C_1 cells express TRH, somatostatin (Tashjian, 1979) and VIP_2 receptors (Rawlings et al. 1995) but are not thought to express functional GHRH receptors or the D_2 receptors normally found in lactotrophs (Zeytin et al., 1984; Bresson et al., 1991). Of the secretin/calcitonin/PTH/PTHrP receptor family only the VIP_2 receptor mRNA is expressed in GH_3 cells (personal communication, E.M.Lutz) and GH_4C_1 cells (Rawlings et al., 1995). cAMP production, phosphoinositide hydrolysis and nitric oxide production mediated by the VIP_2 receptor in GH_3 cells were investigated.

VIP receptors have long been associated with relaxation of smooth muscle, in the gut, airway and the vasculature. Nitric oxide is also associated with the regulation of blood flow and arterial pressure (Umans, 1995). Murthy & Makhlouf (1994) described VIP receptor-mediated activation of nitric oxide synthase(NOS) in dispersed gastric smooth muscle cells providing direct evidence of nitric oxide production as a result of VIP receptor activation. An isoform of constitutive NOS

has been cloned from GH_3 cells (Wolf & Gatto, 1992) making it an appropriate cell line to investigate VIP_2 receptor-mediated stimulation of NOS.

Radioimmunoassay and immunohistochemical methods have shown VIP innervation of cerebral blood vessels (Larsson et al., 1976; Edvinsson et al., 1981). VIP caused vasodilation of isolated cerebral artery or vein upon direct application and increased cerebral blood flow when applied intra-arterially to baboons (Edvinsson et al., 1981). In rat, PACAP and VIP have similar potencies for decreasing systemic arterial pressure (Nandha et al., 1991; Absood et al., 1992) and relaxing tail or mesenteric arteries (Absood et al., 1992; Huang et al., 1993). The ability of PACAP-27, PACAP-38 and VIP to stimulate adenylate cyclase in rat cerebral microvessels has also been demonstrated (Huang & Rorstad, 1983; Kobayashi et al., 1994). Anzai et al. (1995) observed similar concentration-dependent relaxations in canine basilar arteries and rat intracerebral arterioles on addition of PACAP-27, PACAP-38 and VIP. It was not known however which receptors were present in blood vessels. In this study RT-PCR was used to identify the VIP-responsive receptors present in a rat cerebral microvessel preparation.

5.2 Specific Methodology

Cells - GH_3 cells were cultured as described in Section 2.2.1. The cells were not allowed to reach confluency in the flask before being passaged and the growth medium was changed every day in order to minimise any autocrine effects.

Animals - COB-Wistar rats were maintained as desribed in Section 2.2.9.

Ligand-binding - Homologous displacement of ligand-binding was carried out on membranes prepared from GH_3 cells, using [^{125}I]helodermin at 37^{o}C for 10 minutes,

as described in Section 2.2.7. GTPγS modulation of ligand binding was also performed on membranes prepared from GH_3 cells using [^{125}I]helodermin as described in Section 2.2.7.

Protein assay - Coomassie protein assay reagent was used to determine the protein level in samples (see section 2.2.8).

Second messenger assays - Intracellular cAMP production over a 10 minute stimulation period was measured in whole cells by radioimmunoassay as described in Section 2.2.5.

[^{3}H]inositol phosphate production was used as a measure of PLC activity. The cells were stimulated for 60 minutes with agonist before separation of [^{3}H]inositol phosphates by anion exchange chromatography as described in Section 2.2.4.

Nitric oxide synthase activity was measured by the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline over a 10 minute stimulation period. [^{3}H]citrulline production was measured by cation exchange chromatography as described in Section 2.2.6.

Microvessel preparations - A crude preparation of cerebral microvessels was prepared from the cerebral cortex of male Cob-Wistar rats (see Section 2.2.9). The cerebral cortices underwent homogenisation, centrifugation and filtering steps in order to isolate the microvessels, the presence of which was confirmed by microscopy (see Section 2.2.10).

RNA extraction - RNA was extracted from microvessel preparations using the RNAzol B isolation of RNA kit (Ambion Inc.: see Section 2.2.11).

Reverse transcriptase-mediated polymerase chain reaction - The Stratagene RT-PCR kit was used to amplify cDNA transcribed from mRNAs extracted from the microvessel preparation. The resultant fragments were separated by agarose gel

electrophoresis and the ethidium bromide-stained bands visualised using the UV transilluminator (see Section 2.2.12). Primers for the rat VIP_1, VIP_2, PACAP and m1/m3 muscarinic receptors were used as detailed below. PCR reactions were set up in 100 µl volumes including 5 µl first strand reaction mix and 15 pmols of each of the primers. The reaction was put through 30 cycles of 94°C (1 min), 57°C (1 min) and extension at 72°C (2 min). After PCR, 10 µl samples were analyzed by electrophoresis (see Section 2.2.12).

m1/m3 muscarinic receptor primers: 37484 [5' GCAACGCCTCTGTCATGAATCT], 37485 [5' CGAGCTGCCTTCTTCTCCTTGA]

VIP_1 receptor primers: 338 [5'CAACAGCGGGGAGATAGACC], 19181 [5'GAAGACCATTTTCACCTGGG]

VIP_2 receptor primers: 9502 [5'GAATGCCGGTTTCATCTGG], 8670 [5'GGAGATGAGTTCCTGGCTTG]

PACAP receptor primers: 32204 [5'CAGAAGCTTCAGTCCCCAGACATGG], 32205 [5'AGTGAATTCCAAAGAGTGGGATGAG]

Data analysis - Curve fitting was performed by the non-linear curve-fitting programme, P-fit (Elsevier Biosoft, Cambridge).

5.3 Results

5.3.1 Ligand binding studies to determine the expression level and characteristics of the endogenous VIP_2 receptor in GH_3 cells

Homologous displacement of [^{125}I]helodermin binding to GH_3 cell membranes

In radioligand-binding experiments, homologous displacement of [^{125}I]helodermin from GH_3 cell membranes (see 'Materials & Methods' section 2.2.7) was used to measure the receptor binding capacity of the system (B_{max}) and the

affinity of the target receptor for helodermin (IC_{50}) using the method of Swillens (Swillens, 1993). [^{125}I]helodermin was used as a radioligand because of its availability (helodermin was iodinated on site), as well as its reported selectivity and affinity at the VIP_2 receptor (Robberecht et al., 1988). Lutz et al. (1993) found helodermin to be equipotent with VIP, PACAP-38 and PACAP-27 for cAMP production mediated by the rat VIP_2 receptor. Available evidence strongly suggests that the relevant receptor expressed in GH_3 cells is exclusively the VIP_2 type. Rawlings et al. (1995) found GH_4C_1 cells to express mRNA for the VIP_2 receptor and not the VIP_1 or PACAP receptors. The presence of the VIP_2 receptor and not VIP_1 or PACAP receptors in GH_3 cells has also been confirmed by RT-PCR (E.M. Lutz, personal communication). Homologous displacement of [^{125}I]helodermin occurred with an IC_{50} value of 0.35±0.09 nM and a B_{max} of 22.06±1.42 fmol/mg protein (see Figure 5.1). The samples contained 197±8 µg of protein/100 µl and 27,178±1030 cpm of a 65 µCi/ml solution of [^{125}I]helodermin was added to each. Specific binding (displaced by 1 µM helodermin) was 678±43 cpm. A standard curve was generated relating cell numbers to protein content of the sample (not shown) which allowed an estimated figure for receptors per cell to be calculated based on one molecule of helodermin binding per receptor. The GH_3 cells were found to express approximately 3,670±280 VIP_2 receptors per cell.

GTPγS modulation of [^{125}I]helodermin binding to the VIP_2 receptor in GH_3 cell membranes

In order to demonstrate the direct interaction of G proteins with the VIP_2 receptor in GH_3 cells and to investigate the possibility of an interaction with a PTx-sensitive G protein, the effect of GTPγS on [^{125}I]helodermin binding to GH_3 cell membranes was investigated. To facilitate the measurement of GTPγS -displacement of specific [^{125}I]helodermin binding, higher specific activity

[^{125}I]helodermin was used. The iodinated peptide was at a stock concentration of 800 μCi/ml as opposed to 65 μCi/ml. The addition of 317,331±2,828 cpm to each sample produced a specific binding (displaced by 1 μM helodermin) of 8,965±440 cpm (see Figure 5.2). A concentration of 10 μM GTPγS caused the dissociation of 69.0±7.8% of the specific binding (6,186±698 cpm). Membranes prepared from cells treated for 16 hours with 100 ng/ml PTx were also used. The maximal GTPγS-stimulated dissociation of [^{125}I]helodermin from PTx-treated cell membranes was 53.5±6.3% of the specific binding (4,800±566 cpm). Despite the curves appearing to be different, the decrease in [^{125}I]helodermin binding stimulated by GTPγS from membranes treated with PTx was only significantly different to the decrease observed from the control membranes at a single point ($P<0.05$, Mann-Whitney U test, n=6).

In summary, it appears that the VIP_2 receptor in GH_3 cells interacts with G proteins as demonstrated by the GTPγS modulation of ligand binding but it is unclear from these data whether there is any sizeable role for a PTX-sensitive G protein.

5.3.2 Agonist-evoked cAMP production mediated by the VIP_2 receptor in GH_3 cells

Concentration-response experiments were carried out for VIP-, PACAP-38-, PACAP-27- and helodermin-evoked cAMP production. A concentration-dependent stimulation of cAMP production was observed for concentrations of VIP from 0.1 nM - 1 μM (see Figure 5.3). The basal level was 3.98±0.64 pmol/ml cAMP and the maximum stimulation 8.49±0.63 pmol/ml (2.13±0.16 fold of basal control stimulation). The EC_{50} value was 2.86±1.04 nM. A concentration-dependent stimulation of cAMP production was also observed for concentrations of PACAP-38 from 0.1 nM - 100 nM. The basal level was 3.90±0.14 pmol/ml cAMP and the

maximum stimulation 9.56±0.65 pmol/ml (2.45±0.17 fold of basal control stimulation). The EC_{50} value was 3.77±1.16 nM. A concentration-dependent stimulation of cAMP production was observed for concentrations of PACAP-27 from 0.1 nM - 100 nM. The basal level was 4.63±0.02 pmol/ml cAMP and the maximum stimulation 9.32±0.54 pmol/ml (2.01±0.12 fold of basal control stimulation). The EC_{50} value was 2.83±0.98 nM. A concentration-dependent stimulation of cAMP production was observed for concentrations of helodermin from 0.1 nM - 100 nM. The basal level was 3.72±0.59 pmol/ml cAMP and the maximum stimulation 8.53±0.41 pmol/ml (2.29±0.11 fold of basal control stimulation). The EC_{50} value was 0.44±0.20 nM.

A clear concentration-dependent stimulation of cAMP production was observed for all these agonists. VIP, PACAP-38, PACAP-27 and helodermin stimulated similar maximal levels of cAMP production. As expected, VIP, PACAP-38 and PACAP-27 were clearly equipotent for cAMP production mediated by the VIP_2 receptor in GH_3 cells. However, helodermin appeared to be slightly more potent than the other agonists. In SUP-T1 human lymphoblast cells, which express the VIP_2 receptor, Robberecht et al. (1988) also observed helodermin to be more potent than VIP for AC stimulation and inhibition of [^{125}I]-acetyl-His^1-VIP binding.

5.3.3 Agonist-evoked [^{3}H]inositol phosphate production mediated by the VIP_2 receptor in GH_3 cells

Concentration response experiments were carried out for VIP- and PACAP-38-evoked [^{3}H]inositol phosphate production mediated by the VIP_2 receptor in GH_3 cells (see Section 2.2.4).

VIP stimulated a concentration-dependent increase in [^{3}H]inositol phosphate production in GH_3 cells over a 30 nM - 3 μM range (see Figure 5.4(a)). The maximal stimulation was 1.48±0.14 fold of basal control and the EC_{50} value 179±103 nM. PACAP-38 also stimulated a concentration-dependent increase in [^{3}H]IP production in GH_3 cells over a 30 nM - 1 μM range (see Figure 5.4(b)). The maximal stimulation was 1.37±0.11 fold of basal control and the EC_{50} value 71±36 nM. In order to assess whether [^{3}H]IP production mediated by the VIP_2 receptor in GH_3 cells was subject to desensitisation, the accumulation of 1 μM PACAP-38-evoked [^{3}H]IP production was measured at various times over an 80 minute period (see Figure 5.5). The maximum response was 1.92±0.04-fold of basal control after 80 minutes. The response appeared to be linear over the first 60 minutes, although time points earlier than 10 minutes would be advantageous in order to identify any early peaks of IP production which may occur.

Despite reports to the contrary, the VIP_2 receptor is clearly able to stimulate PLC as well as AC activity in GH_3 cells. VIP and PACAP-38 had similar EC_{50}s for [^{3}H]IP production and, as observed in COS 7 cells, the time-dependent accumulation of [^{3}H]IP production mediated by the VIP_2 receptor was not subject to any rapid desensitisation process.

5.3.4 Agonist-evoked [^{3}H]citrulline production mediated by the VIP_2 receptor in GH_3 cells

The VIP_2 receptor has been associated with nitric oxide (NO) production in certain cell types. The L-arginine to L-citrulline conversion method of measuring NO production was used (see Section 2.2.6) to investigate the stimulation of nitric oxide synthase (NOS) mediated by the VIP_2 receptor in GH_3 cells.

The calcium ionophore, ionomycin, was used to demonstrate the ability of Ca^{2+}-influx to stimulate NOS activity in GH_3 cells (see Figure 5.6). At a concentration of 10 μM, ionomycin caused a stimulation of NOS activity equivalent to an increase of 11,971±1092 dpm/assay over the basal value of 16,124±379 dpm/assay. The non-specific G protein-activator, $AlCl_3$/NaF, caused a stimulation equivalent to 50±2% (5,986±215 dpm/assay) of the ionomycin-stimulated response. PACAP-38 at a concentration of 300 nM elicited a response equivalent to 36±7% (4310±778 dpm/assay) of the ionomycin stimulated-response. PACAP-38 at a concentration of 300 nM when applied with 10 μM ionomycin appeared to cause no increase in [^{3}H]citrulline production over that stimulated by 10 μM ionomycin alone (96±4% of the ionomycin-stimulated response; 11,492±479 dpm/assay). A concentration-response experiment was carried out to investigate the characteristics of VIP-evoked [^{3}H]citrulline production in GH_3 cells. VIP caused a concentration-dependent increase in [^{3}H]citrulline production over the concentration range 1 - 300 nM (see Figure 5.7). The maximal stimulation was 1.50±0.04 fold of basal control. The EC_{50} value was 24.0±12.8 nM. As a means of confirming that the agonist-evoked [^{3}H]citrulline production was due to NOS activity, the effect of the NOS inhibitor, N-nitro-L-arginine (L-NNA), was tested on 1 μM VIP-evoked [^{3}H]citrulline production in GH_3 cells (see Figure 5.8). Typical basal values were 18,139±1,480 dpm/assay and the 1 μM VIP-evoked response 1.36±0.01 fold of basal control. L-NNA at concentrations of 10 and 100 μM caused a slight decrease in basal levels of [^{3}H]citrulline production but this decrease was not statistically significant (Mann-Whitney *U*-test). Treatment of the cells with 10 μM L-NNA inhibited 1 μM VIP-evoked [^{3}H]citrulline production (0.76±0.06 fold of basal control) as did 100 μM L-NNA (0.73±0.01 fold of basal control).

In order to determine whether cAMP-dependent protein kinase(PKA) had a role to play in VIP-evoked NO production, the cells were treated with the PKA

inhibitor, KT-5720 (see Figure 5.9). At a concentration of 1 μM, KT-5720 had no effect on basal [^{3}H]citrulline production (0.96±0.10 fold of basal control). 1 μM VIP stimulated a 1.34±0.03 fold of basal control response which became 1.01±0.07 fold of basal control stimulation after treatment of the cells with 1 μM KT-5720 ($p<0.05$, Mann-Whitney *U*-test).

The VIP_2 receptor in GH_3 cells has therefore been demonstrated to be coupled to NOS stimulation. This stimulation is specifically blocked by an NOS inhibitor (L-NNA). Ionomycin treatment demonstrated that calcium influx alone is sufficient to stimulate NOS activity. G protein activation by $AlCl_3$/NaF also stimulates NOS activity. PACAP-38 and ionomycin treatment of cells did not produce any evidence of an additive effect or synergism suggesting that they do not represent separate mechanisms for stimulating NOS. PKA appears to play a positive role in VIP-stimulated NOS activity. It would be of interest to determine whether PLC, Ca^{2+} and/or PKC have a role to play in the regulation of VIP-stimulated NOS activity.

5.3.5 VIP receptors in the cerebral vasculature

A number of pharmacological and binding studies have provided evidence of the presence of VIP receptors in the cerebral vasculature. In order to identify which specific receptors are present RT-PCR was performed (see Section 2.2.11 and 2.2.12) on the mRNA extracted from a crude microvessel preparation from rat cerebral cortex as described in Section 2.2.10.

The results clearly show the presence of mRNA for both the VIP_1 and VIP_2 receptors in this preparation. There was strong evidence for the presence of m1– and m3-muscarinic receptors in human and bovine brain microvessels (Linville & Hamel, 1995). Primers for these receptors were therefore used as a positive control

and the presence of mRNA for the muscarinic receptors is clearly demonstrated in the microvessel preparation (see Fig. 5.10).

5.4 Discussion

The VIP_2 receptor is an important regulator of pituitary gland function and its presence in the GH_3 clonal cell line (as the only VIP/PACAP receptor) provides a valuable opportunity to study VIP_2 receptor-mediated signalling in its native environment. In such conditions the receptor is not over-expressed and the G protein complement should be appropriate to its function.

5.4.1 VIP and the anterior pituitary

VIP has been shown to stimulate cAMP production in GH_3 cells (Gourdji et al., 1979; Onali et al., 1983). VIP stimulates pituitary prolactin release *in vivo* in humans (Kato et al., 1984) and rats (Kato et al., 1978) and *in vitro* in cultured normal human pituitary tissue (Marlarkey et al., 1981), normal rat hemipituitaries (Enjalbert et al., 1980) and in the GH_3 cell line (Gourdji et al., 1979; Onali et al., 1983). A physiological role for VIP and PACAP in anterior pituitary cell function is likely since immunoreactive VIP and PACAP are present in the neurons of the hypothalamic PVN that project to the external zone of the median eminence (Mezey & Kiss, 1985; Koves et al., 1991) and VIP immunoreactivity in hypophysial portal blood is considerably greater than in peripheral blood (Said & Porter, 1979). VIP concentrations measured by RIA in the hypophysial portal blood after hypophysectomy were 0.4 nM - 0.52 nM whereas the VIP concentration in peripheral plasma was less than 0.03 nM in most animals (Shimatsu et al., 1981).

5.4.2 GH_3 cells

GH_3 cells, are an immortalised clonal cell line, that has both somatroph and lactotroph characteristics. Boockfor et al. (1985) found that GH_3 cell cultures contained twice as many GH secreting (somatotroph-like) cells as PRL-secreting (lactotroph-like) cells and that this was dependent on the influence of autocrine and other growth factors. GH_3 cells do not express the dopamine receptors (D_2) normally found in lactotrophs and may not express functional GHRH receptors typical of somatotrophs either (Zeytin et al., 1984; Bresson et al., 1991). Missale et al. (1994) found that nerve growth factor(NGF) treatment caused the differentiation of GH_3 cells into lactotrophs as opposed to somatotrophs. The differentiated cells expressed the lactotroph-specific D_2 dopamine receptor and the spontaneous secretion of PRL was increased in these cells whereas GH secretion was inhibited.

The VIP_2 receptor in GH_3 cells

Bjoro et al. (1987) investigated GH_4C_1 cells and found VIP receptors present at the level of 9,000 per cell. TRH receptors were present at the level of 135,000 per cell in GH_3 cells (Hinkle & Tashjian, 1974) and bombesin receptors at the level of 3,600 per cell (Westendorf & Schonbrunn, 1983). In this study, homologous displacement of [^{125}I]helodermin binding produced an estimate of 3,700 receptors per cell in the GH_3 cell line (see Figure 5.1). Initially VIP was believed to be of hypothalamic origin (Emson et al., 1978; Simms et al., 1980) but it has also been found to be synthesised within the rat anterior pituitary gland (Arnaout et al., 1986; Segerson et al., 1989). There is also evidence that VIP modulates basal PRL release in an auto- or paracrine manner in normal pituitary and GH_3 cells (Hagen et al., 1986; Nagy et al. 1988). It is therefore possible that VIP could exert an autocrine effect on the expression level of VIP receptors and aspects of their signalling behaviour. The conditions under which the cells were cultured were designed to minimise any autocrine effects on VIP_2 receptor expression and function but this is

not to say that the expression level could not be increased by altering these conditions. It is interesting to note that the VIP_2 receptor mRNA level in the pituitary is significantly higher in pregnant rats than in normal females (Usdin et al., 1994) suggesting that VIP_2 receptor-mediated stimulation of prolactin release is physiologically important and that the receptor number is indeed increased *in vivo* by the appropriate physiological cues.

5.4.3 Mechanisms of VIP_2 receptor-stimulated hormone secretion

VIP has been observed to stimulate cAMP production in pituitary cells (Robberecht et al., 1979; Borghi et al., 1979; Onali et al., 1981) and VIP-stimulated prolactin release in GH_3 cells is thought to occur as a result of this AC activation. Tixier-Vidal & Gourdji (1981) identified a role for adenylate cyclase and PKA in VIP-stimulated prolactin release in pituitary cells. Gourdji et al. (1979) however, found that VIP and TRH had an additive effect on prolactin secretion from GH_3 cells whereas TRH had no effect on cAMP production, indicating that TRH stimulates prolactin secretion by a mechanism other than AC stimulation. VIP was also found to promote a pattern of phosphorylation in rat pituitary cell lines that was distinct from that stimulated by TRH and similar to that stimulated by dibutyryl cAMP, 8-bromo-cAMP or cholera toxin treatment (Drust et al., 1982; Sobel & Tashjian, 1983). These phosphorylated proteins are presumed to be involved in the mechanism of prolactin release.

TRH was not thought to cause AC activation but Paulssen et al. (1992) found that TRH weakly stimulated cAMP production in GH_3 cells. The relevant aspect of TRH receptor-mediated signalling however is likely to be something other than AC stimulation, since it is primarily a PLC-stimulating receptor. Of the six prolactin secretagogues (VIP, TRH, bombesin, epidermal growth factor(EGF), insulin and phorbol-12,13-dibutyrate(PDBu)) tested by Dorflinger & Schonbrunn (1983) on

GH_4C_1 cells only VIP raised cAMP levels. Onali et al. (1983) also concluded that there was probably a cAMP-independent mechanism at work and evidence of a stimulatory cAMP-independent mechanism was also provided by Coleman & Bancroft (1993) who found that prolactin gene promoter activity was stimulated by PACAP without any detectable rise in cAMP production. Lin et al. (1996) identified a synergistic activation of cAMP response element-mediated prolactin gene expression by Ca^{2+} and cAMP in GH_3 cells.

The levels of agonist-evoked VIP_2 receptor-mediated cAMP production in GH_3 cells were very much lower than the equivalent levels in COS 7 cells (see Chapter 3 and Figure 5.3), Gourdji et al. (1979) however, observed VIP-evoked cAMP production levels in GH_3 cells at similar levels to those described in this study. The EC_{50}s for cAMP production mediated by the VIP_2 receptor in GH_3 cells were slightly higher than those for cAMP production in COS 7 cells. This is to be expected since the G protein isoforms present and ratio of receptors to G proteins may well be very different.

The possibility of a cAMP-independent pathway being involved in the regulation of agonist-evoked VIP_2 receptor-mediated prolactin secretion from GH_3 cells is also of interest since it has been demonstrated in COS 7 cells that this receptor is capable of stimulating PI hydrolysis and Ca^{2+} channel activity as well as cAMP production (see Chapter 3). Rawlings et al. (1995) observed PACAP-evoked cAMP production and Ca^{2+} oscillations but no stimulation of PI hydrolysis in GH_3 and GH_4C_1 cells. However the authors make the point that the methods used for measuring Ca^{2+} fluctuations are much more sensitive than those for measuring inositol phosphate production. The present investigation has demonstrated a modest stimulation of PLC activity mediated by the VIP_2 receptor in GH_3 cells that is not dependent on cAMP production (see Figure 5.4). This response may have a

role to play in the modulation of hormone release from the pituitary and clonal cell lines through the activation of PKC and/or an increase in intracellular Ca^{2+} concentration and may also be important *in vivo* in other cell types.

Release-inducing hormones are thought to cause negligible inhibition of AC (Gordeladze et al., 1988; Offermanns et al., 1989). The activation of receptors which have an inhibitory influence on VIP-stimulated prolactin secretion tend to reduce cAMP production e.g. the somatostatin receptor reduces cAMP production and inhibits VIP-stimulated prolactin release (Chen et al., 1993) and Van Chuoi et al. (1993) have shown that GnRH-associated peptide(GAP) inhibits VIP-stimulated Ca^{2+} transients and cAMP production via a PTx-sensitive G protein and thereby inhibits VIP-stimulated prolactin secretion from GH_3 cells. G_{o1}, G_{o2}, G_{i2} and G_{i3} have all been detected in GH_3 cell membranes (Rosenthal et al., 1988; Offermanns et al., 1991; Paulssen et al., 1992). Kleuss et al. (1991) identified G_{o2} as the G protein activated by the somatostatin receptor in GH_3 cells, whereas Paulssen et al. (1992) identified the G protein as most likely being G_{i2}. G_{i2} is thought to be involved in receptor-mediated inhibition of AC (Simonds et al., 1989) and has been reported to be a target for PKC (Katade et al., 1985).

Secretion of hormones from pituitary cells is believed to be reliant on Ca^{2+} influx which can be acheived by a number of mechanisms. The LHRH, TRH, AT-II and vasopressin receptors all stimulate PI hydrolysis and hormone secretion from pituitary and other cell lines as a result of $[Ca^{2+}]$ elevation. L-type Ca^{2+} channels have been shown to be directly phosphorylated by both PKA and PKC (Curtis & Catterall, 1985; Nunoki et al., 1989). Armstrong & Kalman (1988) have shown that this facilitates the activation of the channels. Gollasch et al. (1993) described TRH receptor-mediated stimulation of L-type Ca^{2+} channels in GH_3 cells as a result of PKC activation (it was blocked by H-7, staurosporine and calphostin C) and an

additional direct activation of the channel by G_{i2} (with a small contribution from G_{i3}). So in this scenario, G_{i2} is implicated in the stimulation of hormone secretion. It is possible that PKC phosphorylation of the channel is required for its activation by G_{i2}. Alternatively, phosphorylation of the G protein itself by PKC might modify its activity such that it gained the ability to interact with and activate the Ca^{2+} channel.

GH release can also be stimulated by GHRH (the GHRH receptor is positively coupled to AC stimulation) and inhibited by somatostatin (Frohman & Jansson, 1986). This mechanism also involves cAMP and an increase in intracellular Ca^{2+} concentration. Increased cAMP levels have been suggested to cause the opening of a Na^{+}-permeable ion channel, the membrane depolarises as a result, and Ca^{2+} enters through voltage-gated Ca^{2+} channels which promotes the release of GH via exocytosis (Lussier et al., 1991; Naumov et al., 1994).

The AC and PLC systems seem to converge by causing the activation of L-type Ca^{2+} channels and stimulating Ca^{2+} influx which in turn facilitates exocytosis and the release of hormone. Ptx-sensitive G proteins are implicated both in the inhibition of hormone secretion, by reducing AC activity, and the stimulation of hormone secretion, by activating L-type Ca^{2+} channels. An interesting question regarding the regulation of hormone secretion in pituitary cells is whether there are different mechanisms negatively regulating hormone secretion stimulated by receptors which are primarily coupled to AC compared to responses of those which are primarily coupled to PLC.

The negative regulation of hormone secretion

As described above, the cAMP-dependent stimulation of hormone secretion can be inhibited by the activation of receptors coupled to inhibitory G proteins (Onali et al., 1983; Kleuss et al., 1991, Van Chuoi et al., 1993). It has been established that the secretion of prolactin from the anterior pituitary is under tonic inhibitory hypothalamic control mediated by dopamine (Ben-Jonathan, 1985). Dopamine or its receptor agonists inhibit the release and the expression of prolactin as well as lactotroph proliferation (Shaar & Clemens, 1974; Pawlikowski et al. 1978). A physiological stimulus such as suckling causes an immediate drop in dopamine levels in the rat anterior pituitary(Chiocchio et al. 1979; Selmanoff & Wise, 1981; Demarest et al., 1983) and this is a requirement for prolactin release (Grosvenor, 1980). Investigations by Balsa et al. (1996) revealed that DA inhibits both the expression of VIP mRNA and the secretion of VIP from anterior pituitary cells thereby preventing the autocrine activity of VIP in stimulating prolactin release. *In vitro* studies have confirmed that prolactin release is stimulated after short term exposure to DA in superperfused hemipituitaries (Fagin & Neill, 1981) and aggregated (Denef et al., 1984) or dispersed (Martinez et al., 1988) anterior pituitary cells. VIP is a well-established prolactin-releasing factor (Kato et al., 1978; Ruberg et al., 1978; Enjalbert et al., 1980), which has also been shown to modulate IGF-I- (Lara et al.,1994) and TRH-stimulated prolactin-release from anterior pituitary cells

The role of nitric oxide

Duvilanski et al. (1995) investigated the stimulation of prolactin release from hemipituitaries by VIP and substance P. These authors found that nitric

oxide synthase(NOS) inhibitors stimulated prolactin release and that the effect was additive with that of VIP. Substance P caused a small increase in prolactin release without being affected by NOS inhibitors. The nitric oxide-donor, sodium nitroprusside(NP), was found to inhibit prolactin release. Dopamine- and atrial natriuretic factor-evoked inhibition of prolactin release was inhibited by NOS inhibitors. LH release was unaffected. NP was found to increase cGMP levels in the anterior pituitary and to reduce cAMP levels. The guanylate cyclase inhibitor, LY 83583, and the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine(IBMX), both blocked the inhibitory effect of NP on prolactin release, the application of 8-bromo-cGMP mimicked the effect of NP on prolactin release. Nitric oxide therefore appears to inhibit prolactin release by stimulating guanylate cyclase activity. The authors attribute the effects of IBMX to the inhibition of cAMP-specific phosphodiesterases(PDEs) and the resultant accumulation of cAMP which is believed to have the opposing effect to cGMP in this system. IBMX however, is an inhibitor of a broad spectrum of PDEs and the authors did not present any data on the effects of IBMX on cAMP and cGMP levels in these cells. Duvilanski et al. (1996) suggest that dopamine may utilise cGMP as a component of the mechanism by which it inhibits prolactin secretion. However those authors showed that it caused only a small increase in NO production and failed to demonstrate that it causes any increase in cGMP levels at all.

Hartt et al. (1995) however found that cGMP was stimulatory for GH release. GH release was stimulated by C-type natriuretic peptide (CNP), which stimulates a membrane-bound form of guanylate cyclase, and NP, a NO donor, which indirectly stimulates soluble guanylate cyclase. No increase in cAMP was observed in response to CNP and intracellular [Ca^{2+}] was slightly reduced. One major difference between these studies is that those which found cGMP to be inhibitory were carried out on hemipituitaries whereas the study by Hartt et al.

(1995) was performed on a static monolayer culture of dispersed pituitary cells. The enzymatic digestion process and dispersal of the cells is likely to have profound effects on the interactions between the pituitary cells.

In this study it has been demonstrated that the agonist stimulation of the VIP_2 receptor in GH_3 cells causes a concentration-dependent increase in NO levels in the cell (approximately 40-50% increase above the basal level at 300 nM VIP: see Figure 5.7). Ionomycin (10 μM) alone caused a 74% increase. Ca^{2+} influx is therefore sufficient to stimulate NO production and PACAP-38 caused no additional stimulation beyond the effect of ionomycin (see Figure 5.6). This suggests that there is no additional potentiatory step mediated by the VIP_2 receptor, such as that described by Murthy & Makhlouf (1994) in gastric smooth muscle cells, and that the response may be entirely dependent on Ca^{2+} influx. The PKA inhibitor, KT-5720 appears to considerably reduce the stimulation of NOS by the VIP_2 receptor indicating that PKA may play a role in the activation of a Ca^{2+} channel and the resultant activation of NOS. In order to fully investigate the influence of cAMP/PKA on VIP_2 receptor-mediated stimulation of NOS in this system a number of additional experiments should be carried out : these should include an investigation of the effects of inhibitors of Ca^{2+}-calmodulin dependent protein kinase, and of PKA, and the effects of forskolin (which acts at the level of AC to stimulate cAMP production) and analogues of cAMP.

Due to the low expression level of the VIP_2 receptor in GH_3 cells and the small stimulation of NOS and PLC activity mediated by the VIP_2 receptor it was not possible to determine whether PTx-sensitive G proteins contributed to these responses or indeed convincingly demonstrate an interaction with the VIP_2 receptor by GTPγS modulation of ligand binding in these cells (see Figure 5.2).

5.4.4 VIP, smooth muscle relaxation and cerebral blood flow

With respect to the cerebral vasculature, a role for neurotransmitters released from the nerves innervating blood vessels in the control of blood flow has long been recognised. VIP innervation of cerebral blood vessels has already been demonstrated by both immunohistochemical and radioimmunoassay studies (Larsson et al., 1976; Edvinsson et al., 1981). Bipolar neurons were identified projecting to local blood vessels in the rat cerebral cortex that stained for either VIP or choline acetyltransferase with only a small proportion staining for both, indicating that there are distinct populations of VIP- and acetylcholine-releasing neurons innervating blood vessels in the cerebral cortex; 'VIP' neurons accounting for the larger portion of the population (Chedotal et al., 1994). The ability of VIP to modulate cerebral blood flow has also been demonstrated on a number of occasions. Huang & Rorstad (1983) showed that only VIP out of 22 peptides tested could stimulate cAMP production in rat cerebral microvessel preparations and identified distinct VIP and PTH receptors in rat cerebral microvessels and arteries. VIP has also been shown to stimulate cAMP production in rat mesenteric artery rings, mesenteric artery smooth muscle and aortic smooth muscle as well as stimulating vasodilation in rat mesenteric artery bed and mesenteric artery and aortic smooth muscle. In rat aortic smooth muscle cells VIP stimulated AC activation with an EC_{50} of 12±6 nM (Ganz et al., 1986). Nandha et al. (1991) demonstrated that PACAP-27 could displace VIP-binding to blood vessel membranes and that PACAP-38, PACAP-27 and VIP administration caused hypotension in the anaesthetised rat. The authors found that VIP and PACAP-38 were comparable in effect, whereas PACAP-27 was three times less potent than VIP. Kobayashi et al. (1994) showed that PACAP-38, PACAP-27 and VIP

stimulated cAMP production in rat cerebral microvessels with EC_{50}s of 7.0, 4.7 and 34 nM respectively and suggested that a PACAP receptor may be present. In the present study, low levels of PACAP receptor mRNA were found to be present in the rat cerebral microvessel preparation (see Figure 5.10). Anzai et al. (1995) observed similar relaxation of canine basilar arteries and rat intracerebral arterioles by VIP, PACAP-27 and PACAP-38 whereas Foda et al. (1995) found PACAP -38 to be one-third as potent and only 70% as efficacious as VIP in stimulating relaxation of guinea pig airway smooth muscle despite PACAP -38 being twice as effective in stimulating AC. Edvinsson et al. (1981) had shown that the direct application of VIP to isolated cerebral arteries or veins produced vasodilation. These authors also showed that increased blood flow could be stimulated in baboons by intra-arterial administration of VIP. Wilson et al. (1981) showed that intracerebroventricular application of VIP caused increased cerebral blood flow in dogs. The likelihood is that VIP receptors in the smooth muscle cells of the blood vessels mediate stimulation of adenylate cyclase, and possibly, nitric oxide synthase, thereby facilitating relaxation of the smooth muscle and vasodilation, although additional mechanisms may be involved.

Foda et al. (1995) examined the effects of PACAP-38 and VIP on the relaxation of isolated perfused guinea pig airway smooth muscle strips. These authors found that PACAP-38 had a more prolonged effect than VIP, probably because of its resistance to enzymatic degradation, and that the relaxation was not entirely due to cAMP production. The VIP_2 receptor has been shown to mediate the stimulation of NO production in GH_3 cells in this study. Murthy & Makhlouf (1995) described the relaxation of dispersed gastric smooth muscle cells as a result of VIP receptor activation. This relaxation was found to have both a cAMP component and a Ca^{2+} influx-dependent NO component. These authors went on to implicate PKA and cGMP-dependent protein kinase in the inhibition of IP_3-

stimulated Ca^{2+} release (1 μM IP_3-treated permeabilised cells) and the stimulation of smooth muscle relaxation. As mentioned above, it is interesting that Anzai et al. (1995) observed PACAP-38, PACAP-27 and VIP to cause similar concentration-dependent relaxation of both canine basilar arteries and rat intracerebral arterioles. In these experiments removal of the endothelium from basilar arteries or treatment with the cyclo-oxygenase inhibitors, aspirin or indomethacin, enhanced the maximum PACAP-27-stimulated relaxation. The prostaglandin, $PGF_{2\alpha}$, or its precursor PGH_2, was implicated as an endothelium-derived constricting factor. The data also indicate that the VIP/PACAP receptor involved is expressed on smooth muscle cells and may additionally be present on the endothelium. An intriguing aspect of this system, is that nitric oxide is known to stimulate cyclo-oxygenase activity (Salvemini et al., 1993). The contrasting actions of nitric oxide are also observed in the uterus where nitric oxide-mediated activation of guanylate cyclase causes relaxation of smooth muscle, and the release of prostaglandins stimulated by nitric oxide causes contraction (Franchi et al., 1994).

Considerable pharmacological evidence for the presence of a VIP receptor in the vasculature has accumulated over the years. In this study the presence of the VIP_1, VIP_2 and a low level of PACAP receptor mRNA in a crude preparation of rat cerebral microvessels has been confirmed by RT-PCR.

5.4.5 Summary

In GH_3 cells the endogenous VIP_2 receptor has been demonstrated to mediate the stimulation of cAMP production, inositol phosphate formation and nitric oxide production. This is in agreement with the data described in Chapter 3 using a heterologous expression system: when expressed in COS 7 cells, the VIP_2 receptor mediates the stimulation of cAMP production and phosphoinositide hydrolysis (COS 7 cells do not appear to express any isoforms of constitutive NOS

so VIP receptor-mediated stimulation of NOS could not be studied in that system). VIP_2 receptor-mediated stimulation of phosphoinositide hydrolysis in COS 7 cell proved to be partially dependent on Ca^{2+} influx and the activity of a PTx-sensitive G protein. The role of PTx-sensitive G proteins in VIP_2 receptor-mediated signalling, as described in Chapter 3 , needs to be explored further in the context of GH_3 cells: an examination of the dependence of PLC and NOS activation on Ca^{2+} influx and PTx-sensitive G proteins would be of great interest. When expressing a receptor in a host cell system it is a concern that expression of the receptor at higher than physiological levels might lead to its coupling promiscuously to various G proteins, the end-result being signalling characteristics that are not physiologically relevant. In GH_3 cells, where the VIP_2 receptor is expressed at low level, the signalling characteristics of the receptor have proven to be very similar to the VIP_2 receptor in COS 7 cells, where it is expressed at a high level.

In the anterior pituitary gland the VIP_2 receptor appears to stimulate prolactin release via cAMP production. Nitric oxide production stimulated by this receptor may act as a negative feedback mechanism. As described above, hormone secretion can be stimulated by both the PLC and AC pathways which appear to converge at the L-type Ca^{2+} channel through the action of PKC and PKA. The primary mechanism of VIP_2 receptor-mediated stimulation of hormone secretion is likely to be cAMP production.

In a rat cerebral microvessel preparation, mRNA for both the VIP_1 and VIP_2 receptors has been shown to be present, in addition to low levels of PACAP receptor mRNA. Nitric oxide production and cAMP production may act as dual arms of a smooth muscle relaxation mechanism as described by Murthy & Makhlouf (1995).

Fig. 5.1 Homologous displacement of [^{125}I]helodermin binding to GH_3 cell membranes.

The binding assay was performed at 37^{o}C for 10 minutes on GH_3 cell membranes. The values are the means±SEM, n=6. [^{125}I]helodermin was present at a concentration of 31.8±1.2 pM. Curve fitting was performed by the non-linear curve-fitting programme P-fit (Elsevier Biosoft, Cambridge, UK).

Fig. 5.2 The effect of pertussis toxin pre-treatment on GTPγS modulation of [^{125}I]helodermin binding to GH_3 cell membranes.

The binding assay was performed at 37^{o}C for 10 minutes on control (□) and PTx-treated (●) GH $_3$ cell membrane preparations. The values are the means±SEM, n=6. For the PTx treatment the cells were pre-incubated for 16 hours with 100 ng/ml PTx before the membranes were prepared. [^{125}I]helodermin was present at a concentration of 30.2±0.3 pM. (*) Represents statistically significant inhibition of GTPγS-mediated dissociation of [^{125}I]helodermin by PTx treatment (p<0.05 by Mann-Whitney U-test).

Figure 5.1

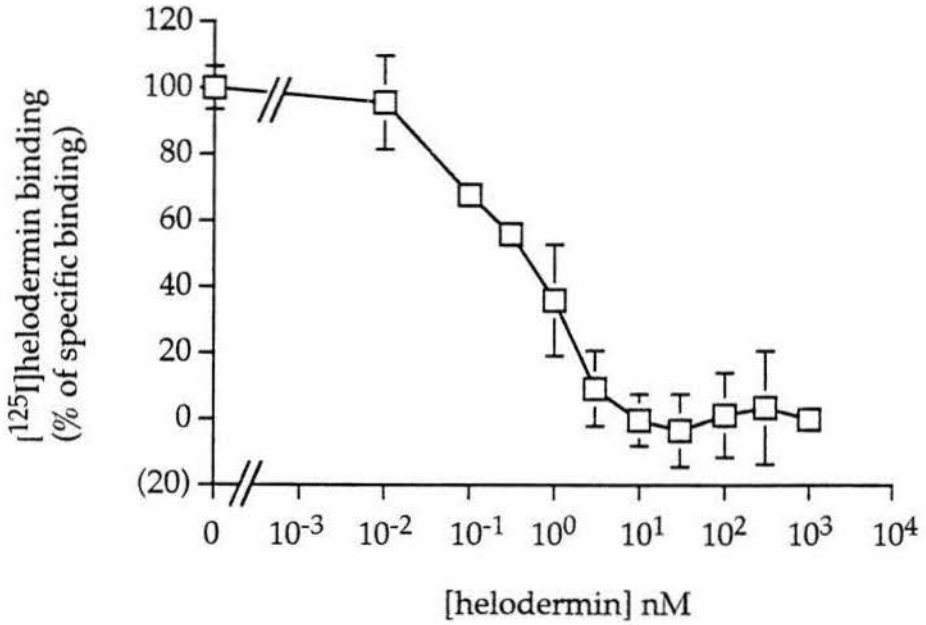


Figure 5.2

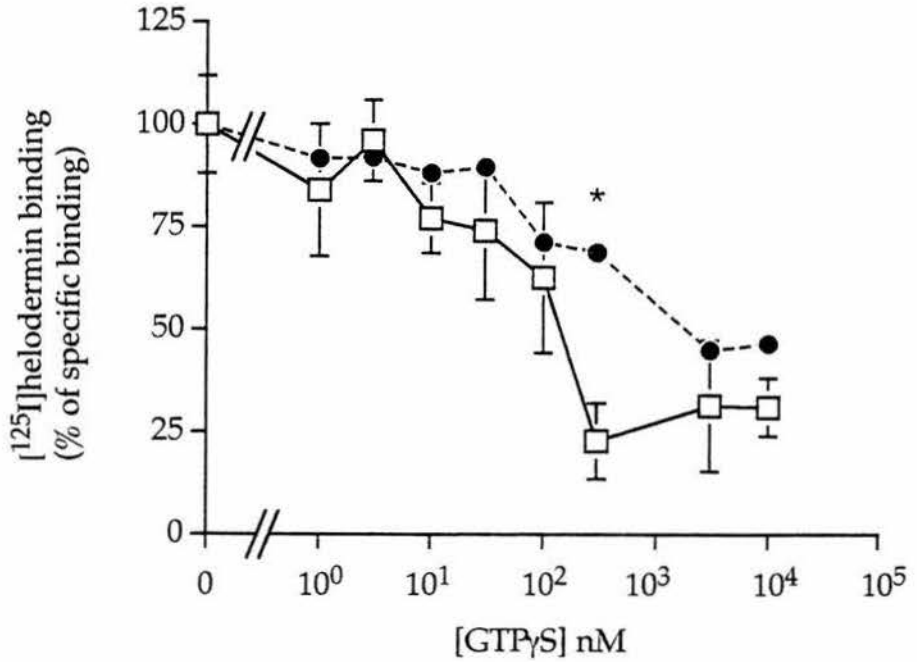


Figure 5.3 Concentration-response curves for agonist-evoked cAMP production in GH_3 cells.

Typical concentration-response curves for VIP (□), PACAP-38 (◇), PACAP-27 (○) and helodermin (△)-evoked intracellular cAMP production in GH_3 cells. The values are means±SEM. n=6.

Figure 5.3

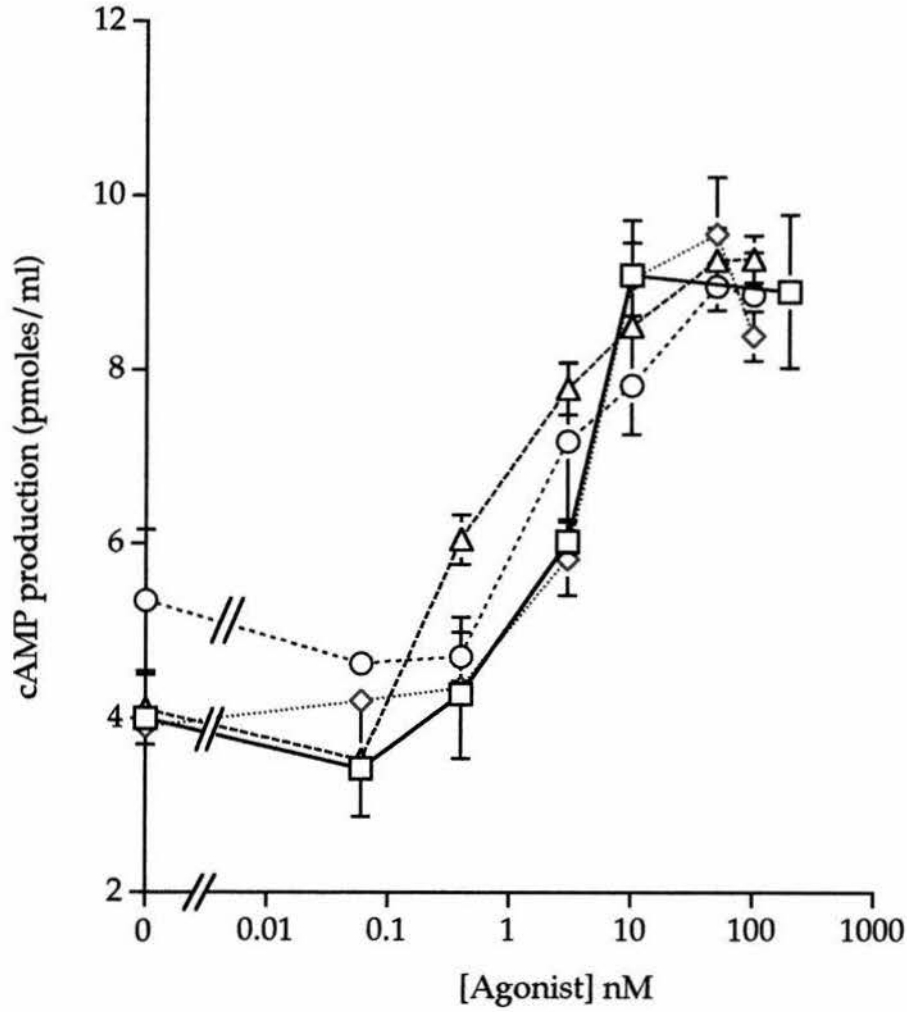
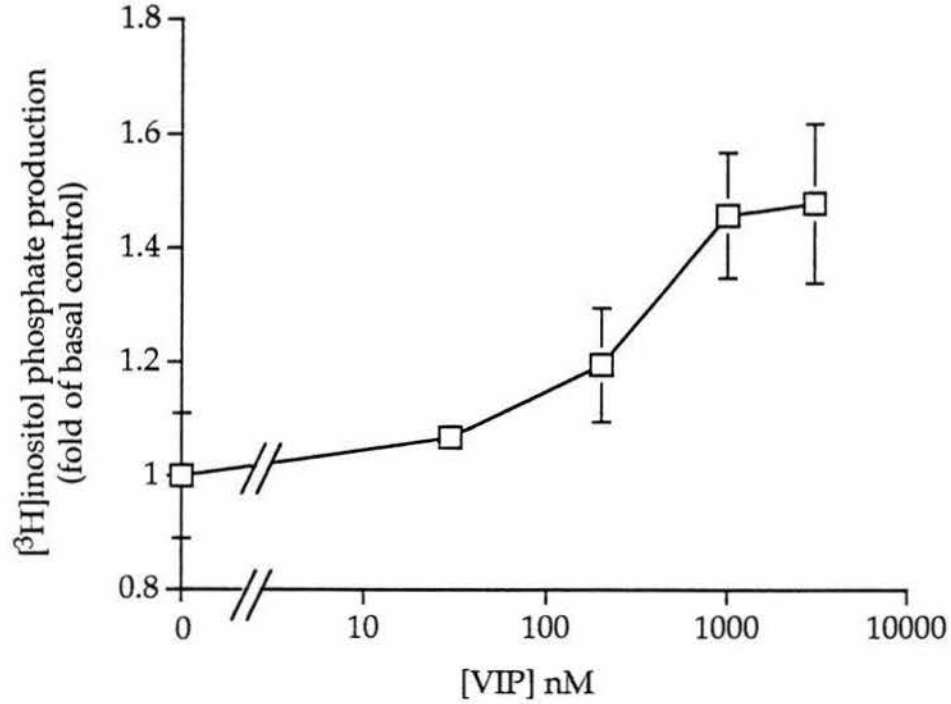

12
10
8
6
4
2
cAMP production (pmoles/ml)
0
0.01
0.1
1
10
100
1000
[Agonist] nM

Figure 5.4 Concentration-response curves for agonist-evoked [^{3}H]inositol phosphate production in GH_3 cells.

a) VIP-evoked [^{3}H]inositol phosphate production. b) PACAP-38-evoked [^{3}H]inositol phosphate production. Typical basal values were 5,329±96 dpm/assay and 4,706±13 dpm/assay respectively. The values are means±SEM. n=6.

Figure 5.4

a)


[3H]inositol phosphate production
(fold of basal control)
1.8
1.6
1.4
1.2
1
0.8
0
10
100
1000
10000
[VIP] nM

b)

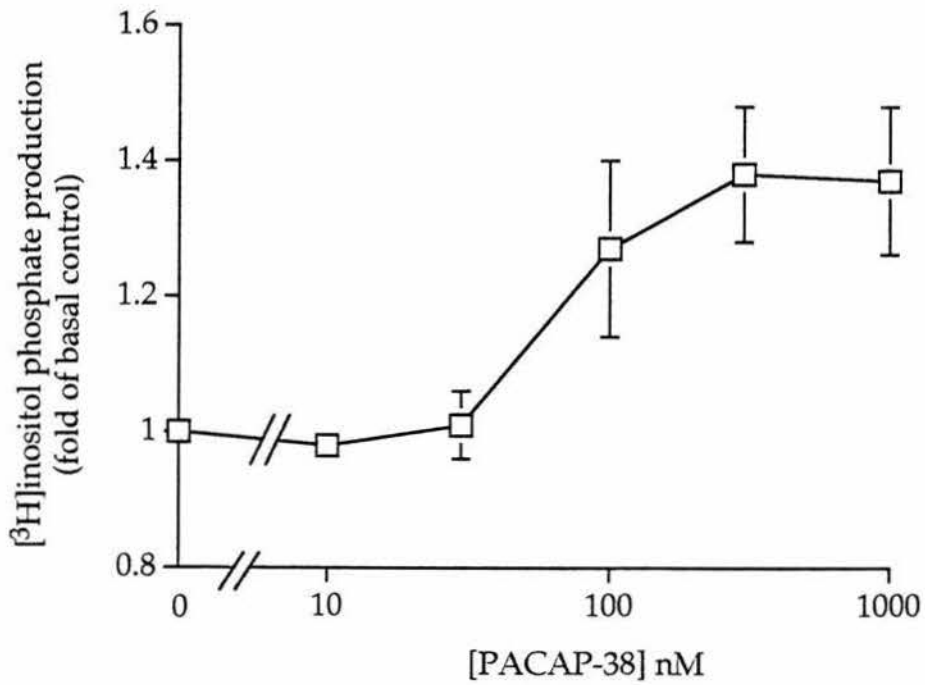

[3H]inositol phosphate production
(fold of basal control)
1.6
1.4
1.2
1
0.8
0
10
100
1000
[PACAP-38] nM

Figure 5.5 Time-course of PACAP-38-evoked [^{3}H]inositol phosphate production in GH_3 cells.

Typical basal values were 4,268±314 dpm/assay. The values are means±SEM. n=6.

Figure 5.5

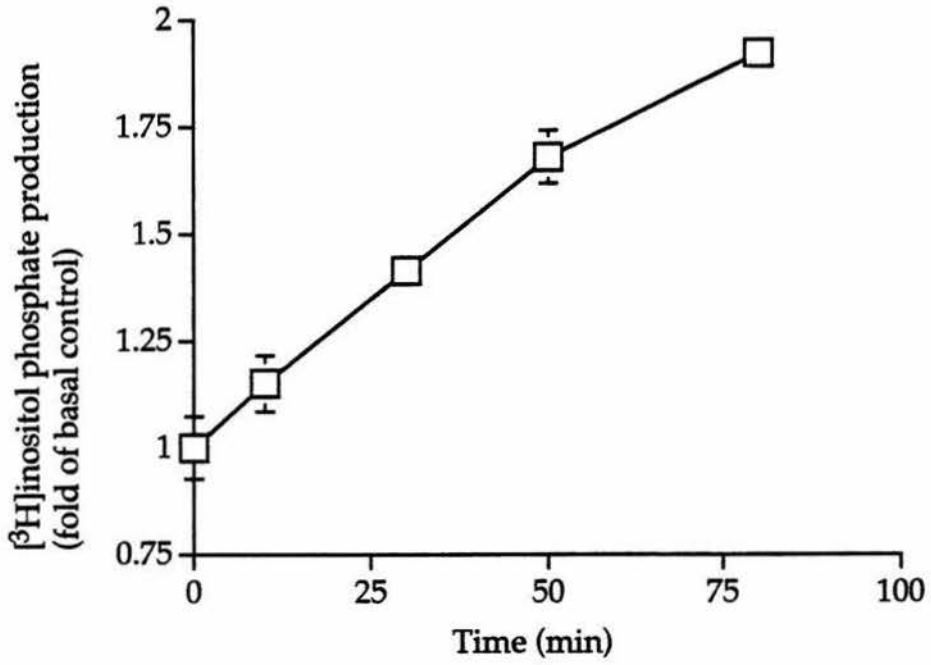

[3H]inositol phosphate production
(fold of basal control)
2
1.75
1.5
1.25
1
0.75
0
25
50
75
100
Time (min)

Figure 5.6 The effects of ionomycin, PACAP-38 and $AlCl_3$/NaF on [^{3}H]citrulline production in GH_3 cells.

The control response was the [^{3}H]citrulline production stimulated by a 10 minute incubation with 10 μM ionomycin (28,095±1025 dpm/assay, the basal value was 16,124±377 dpm/assay). PACAP-38 was applied at a concentration of 300 nM, $AlCl_3$ at 10 μM and NaF at 50 mM. The data are means±SEM. n=6-8.

Figure 5.7 Concentration-response curve for VIP-evoked [^{3}H]citrulline production in GH_3 cells.

The values are means±SEM. n=6. Curve-fitting was performed by the non-linear curve-fitting programme, P-fit (Elsevier Biosoft, Cambridge, UK).

Figure 5.6

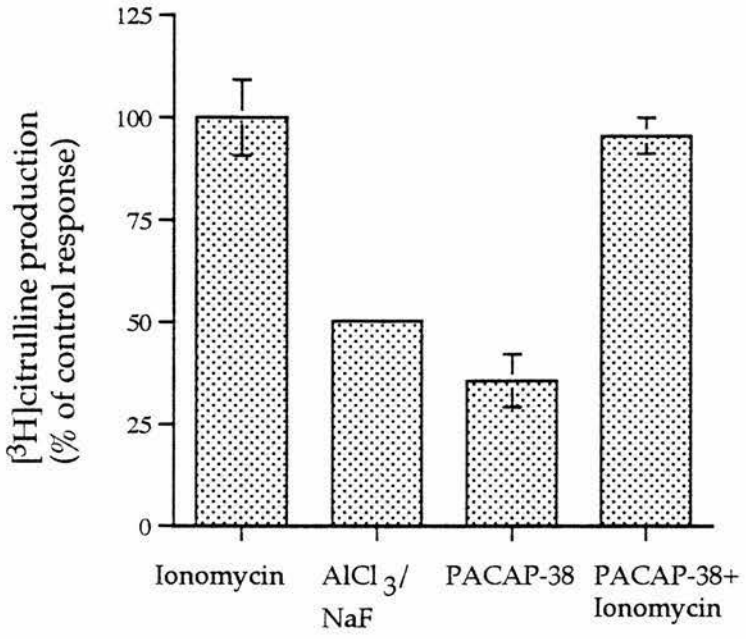


Figure 5.7

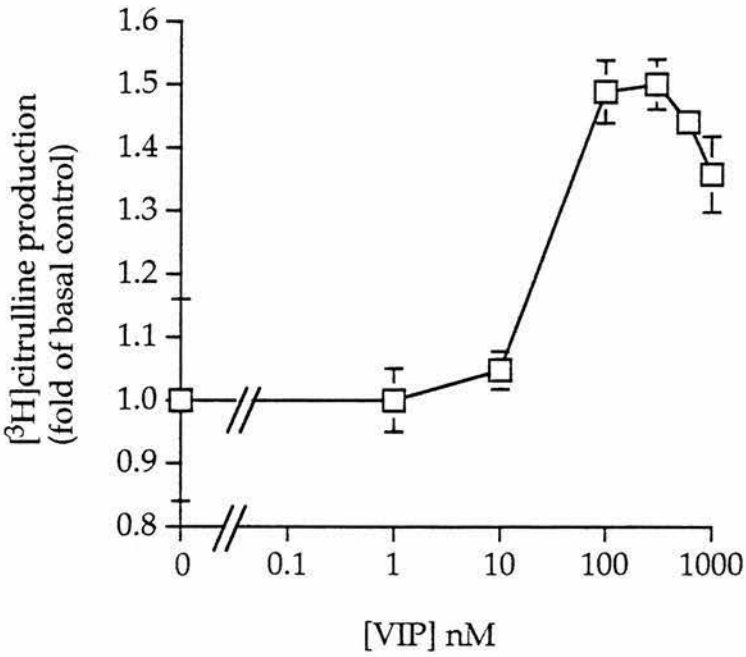


Figure 5.8 The effect of N-nitro-L-arginine, the nitric oxide synthase inhibitor, on VIP-evoked [^{3}H]citrulline production in GH_3 cells.

N-Nitro-L-arginine was applied for 30 minutes prior to stimulation with VIP. The data are means±SEM. n=6.

Figure 5.9 The effect of KT-5720, the protein kinase A inhibitor, on VIP-evoked [^{3}H]citrulline production in GH_3 cells.

The KT-5720 was applied for 30 minutes prior to stimulation with VIP. The data are means±SEM. n=6. (*) Represents statistically significant inhibition of VIP-evoked [^{3}H]citrulline production by KT-5720 treatment ($p<0.05$ by Mann-Whitney *U*-test).

Figure 5.8

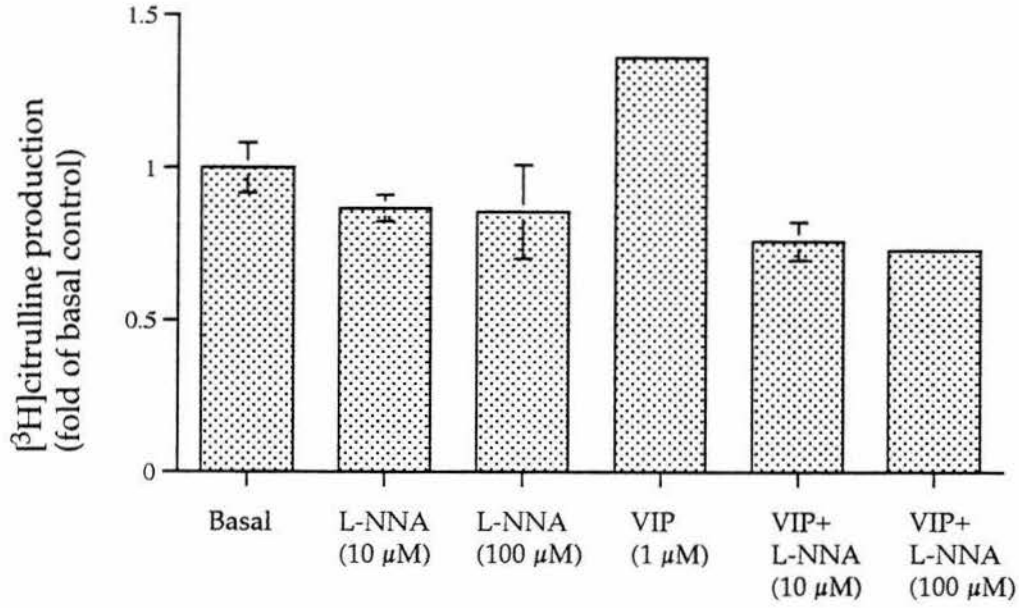


Figure 5.9

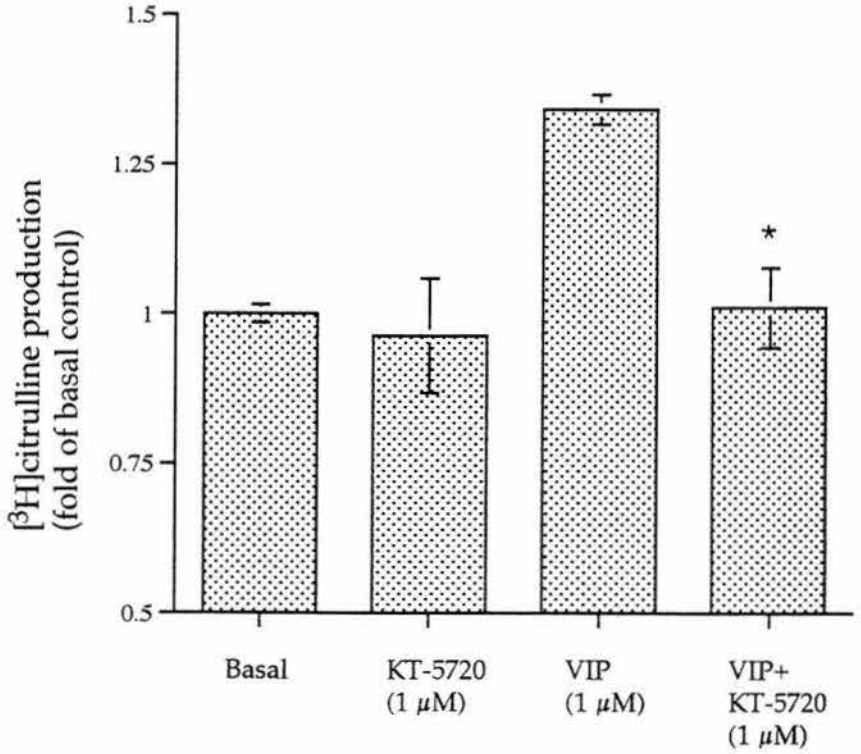
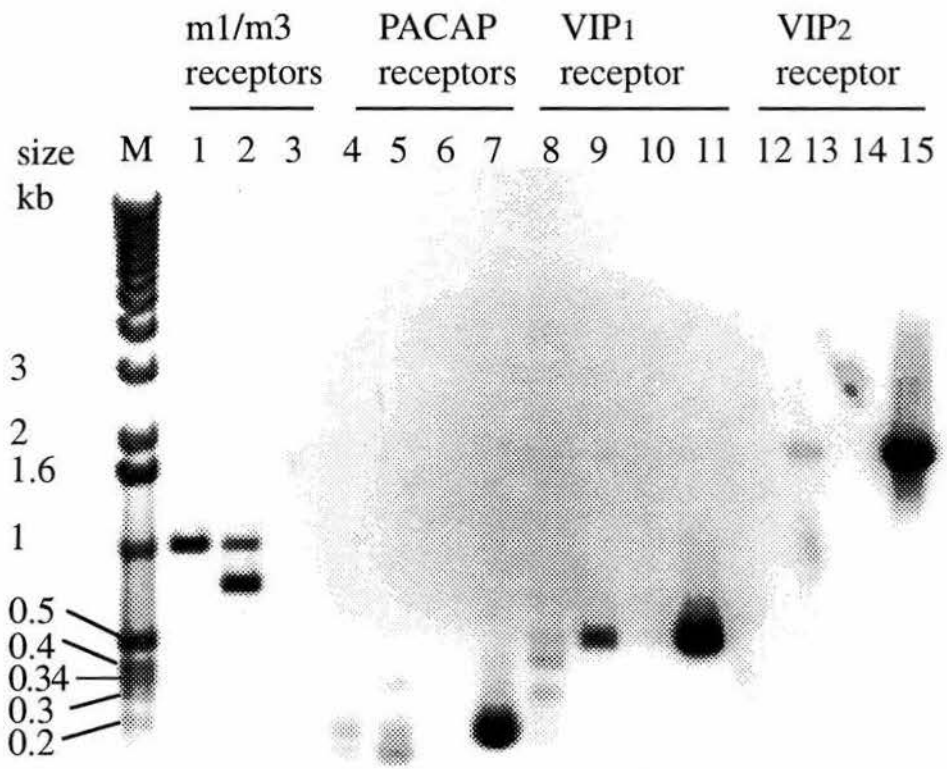


Figure 5.10 Gel electrophoresis of RT-PCR products in order to determine which receptors are expressed in rat brain microvessels

After 30 cycles of amplification, 10 µl of each reaction was run on an 0.7% agarose (BRL, Paisley, UK) gel in 1x Tris/acetate/EDTA buffer (see Section 2.2.12). The molecular weight markers (1 kb DNA ladder, BRL, Paisley, UK) were run in the lane marked M, the m1/m3 receptor primer reactions were run in lanes 1-3, the PACAP receptor primer reactions in lanes 4-7, the VIP_1 receptor primer reactions in lanes 8-11 and the VIP_2 receptor primer reactions in lanes 12-15. The cDNAs for the VIP_1 (PV19), the VIP_2 (6.3) and the PACAP receptors (9.1: encoding the long IC3 splice variant of the PACAP receptor) were used as positive controls. The sizes of DNA fragments in the molecular weight marker lane are listed along the lefthand side of the figure. The m1/m3 receptor primers amplified two fragments which correspond to the sizes expected for the m1 receptor (759 bp) and m3 receptor (1018 bp) in the microvessel extract (lane 2) whilst only the 1 kb fragment was detected in the brain extract (lane 1). In previous reactions, both fragments were detected in the brain preparation. The PACAP receptor primers amplified two fragments which correspond to the sizes expected for the short form (100 bp) and the 84 bp intracellular loop 3 splice variant (184 bp) of the PACAP receptor in both microvessel (lane 5) and brain (lane 4) extracts. Both the VIP_1 (lane 9) and VIP_2 (lane 13) receptor primers amplified fragments which corresponded to the expected size (520 bp and 1844 bp respectively) for each set of primers from microvessel extract. Only a very faint band could be detected for the VIP_1 receptor in brain extract (lane 8).

Lane	RNA Sample	Primers used for reaction
1	Brain extract	m1/m3 receptors
2	Microvessel extract	m1/m3 receptors
3	Negative control	m1/m3 receptors
4	Brain extract	PACAP receptors
5	Microvessel extract	PACAP receptors
6	Negative control	PACAP receptors
7	Positive control	PACAP receptors
8	Brain extract	VIP_1 receptor
9	Microvessel extract	VIP_1 receptor
10	Negative control	VIP_1 receptor
11	Positive control	VIP_1 receptor
12	Brain extract	VIP_2 receptor
13	Microvessel extract	VIP_2 receptor
14	Negative control	VIP_2 receptor
15	Positive control	VIP_2 receptor

Figure 5.10


m1/m3 receptors
PACAP receptors
VIP1 receptor
VIP2 receptor
size kb
M
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15
3
2
1.6
1
0.5
0.4
0.34
0.3
0.2

Chapter 6

Overview

Early studies on VIP receptor function in native tissues were hampered by the problems of distinguishing different receptor types using pharmacological methods: PACAP was isolated (Miyata et al., 1989) nineteen years before VIP was discovered (Said & Mutt 1970) and many studies were performed without, what would now be considered, the most basic pharmacological characterisation of the observed responses i.e. determining the relative potencies of VIP, PACAP-38 and PACAP-27. Many studies are still carried out without sufficient characterisation of the responses, a number of interesting VIP and PACAP-evoked physiological reponses have therefore been observed without being convincingly attributed to the activity of specific receptor types. In recent years the lack of selective antagonists has been the main impediment to identifying the physiological roles of specific receptors. A few 'selective agonists' such as the lizard peptide helodermin (which was thought to be selective for the VIP_2 receptor over the VIP_1 or PACAP receptors (Robberecht et al., 1988)) were available, but did not prove to be equally selective under all conditions, the same was generally found to be true of the available 'selective antagonists'. It is also unfortunate that the characterisation of responses is still complicated by an artificial division that exists between the those who work on VIP and and those who work on PACAP.

The breakthrough in this field came with the molecular cloning of the VIP_1, VIP_2 and PACAP receptors (Lutz et al., 1993; Ishihara et al., 1992; Sreedharan et al., 1993; Adamou et al., 1995; Morrow et al., 1993; Ogi et al., 1993; Ohtaki et al., 1993). This meant that the receptor of interest could be expressed in a heterologous expression system and its responses and interactions studied in isolation from the other VIP- and PACAP-responsive receptors. The distribution of specific receptors in tissue could then also be detected by Northern blotting, *in situ* hybridisation or RT-PCR.

In this study the rat VIP_2 receptor (first cloned by Eve Lutz in this laboratory) and the two splice variants of the PACAP receptor most commonly expressed in mammalian tissue (a long form with a 28 amino acid insert in IC3 and short form without the insert: Morrow et al., 1993) were expressed transiently in COS 7 cells, as were a series of chimaeric VIP_2/PACAP receptors created by Eve Lutz in this laboratory. The rat VIP_2 and PACAP receptors are 51% homologous at the amino acid level yet they have distinct pharmacological characteristics (Lutz et al., 1993), the homology between the receptors was exploited in the creation of the chimaeric receptors (see Fig. 4.9 and Table 4.3) in order to investigate the role of different receptor domains on the VIP_2 receptors signalling characteristics. The N-terminal domain of the VIP_2 and PACAP receptors was identified as the major determinant of ligand specificity, in chimaeric receptor V_1P, the VIP_2 receptor N-terminal domain proved sufficient to cause a major increase in the potency of VIP at the PACAP receptor while having little effect on the PACAP-38-evoked signalling, whereas the PACAP receptor N-terminus prevented VIP-evoked VIP_2 receptor-mediated PLC stimulation and greatly decreased the potency of VIP for AC stimulation. For the secretin, calcitonin, PTH and VIP receptors, agonist binding has been found to be dependent on residues at multiple sites through the receptor's N-terminal domain and extracellular regions (Gourlet et al., 1996; Holtmann et al., 1995a; Holtmann et al., 1995b; Holtmann et al., 1996a; Holtmann et al., 1996b; Nicole et al., 1997; Knudsen et al., 1997; Turner et al., 1996). In this study we found that the N-terminus is crucial for determining the ligand-binding specificity of the VIP and PACAP receptors.

It would be of considerable interest in the future to define any significant differences that may exist between the activities of the VIP_1 and VIP_2 receptors. Based on the differences in their amino acid sequences and the fact that they co-exist in the same cell types it is reasonable to assume that differences do exist.

However, it is possible that the difference between the receptors lies entirely in their specific ligand interactions: helodermin was found to be more potent than VIP at the VIP_2 receptor in SUP-T1 lymphoblast cells (Robberecht et al., 1988) and less potent than VIP at the VIP_1 receptor expressed in COSGs1 cells (Ishihara et al., 1992), secretin was found to be less potent than VIP at the VIP_1 receptor, expressed in COSGs1 cells (Ishihara et al., 1992), but was inactive at the VIP_2 receptor, expressed in COS 7 cells (Lutz et al., 1993). There is therefore evidence of a difference in ligand specificity between the VIP_1 and VIP_2 receptors which has yet to be shown to be physiologically relevant.

In this study we have shown that the VIP_2 receptor can mediate the activation of NOS in GH_3 cells. Similarly Murthy & Makhlouf (1995) showed that a VIP receptor in gastric smooth muscle was coupled to NOS activation. (there is no evidence that a PACAP receptor can activate NOS). We have also established that the VIP and PACAP receptors stimulate PLC by different mechanisms and to different extents. In view of their different signalling characteristics it would be of interest to determine whether any cross-talk occurs between PACAP and VIP receptors especially since they have ligands in common and are sometimes co-expressed in the same cell types. The response of neighbouring cell types in tissue to the same ligand, e.g. PACAP-38 or PACAP-27, may be dependent on the combination of VIP_1, VIP_2 and PACAP receptor splice variants expressed in the cell.

The activation of VIP family receptors has been found to have a synergistic effect on the activity of a number of other receptor types. The VIP receptor in various tissues has been shown to potentiate glucose-induced insulin secretion (Bertrand et al., 1996) and there are several examples now of synergism between α_1-adrenergic receptor and VIP receptor activities; causing an increase in the

magnitude of a Ca^{2+} signal in astrocytes from rat cerebral cortex (Fatatis et al., 1994), stimulating pineal *N*-Acetyltransferase activity (Yuwiler et al., 1987) and potentiating VIP-evoked cAMP and cGMP production in pinealocytes (Chik et al., 1998a; 1998b). It seems unlikely that many physiological responses are truly attributable to the activity of a single receptor stimulated in isolation, synergistic interactions are therefore an aspect of VIP receptor activity that deserves further attention and may prove to be an important general mechanism for modulating the receptors function.

Studies in native cells have suggested that there may be a physiological role for VIP receptor-mediated PLC activation. VIP-stimulated PI hydrolysis was observed in superior cervical ganglion (Audigier et al., 1986) and adrenal chromaffin cells (Malhotra et al., 1988), VIP augmented α-adrenoreceptor-mediated PI hydrolysis and release of calcium from intracellular stores in type 1 astrocytes from rat cerebral cortex (Fatatis et al., 1994), and stimulated calcium release in astroglial cells from rat cerebral cortex (Russell et al., 1990). Low VIP concentrations were also sufficient to activate PKC in regions of rat hippocampus (Weill et al., 1989) and signifigantly stimulation of the mouse VIP_2 receptor expressed in *Xenopus* oocytes caused the activation of calcium-activated chloride currents suggesting that intracellular calcium stores were being emptied. It is in this study however that it has been directly demonstrated for the first time, that the rat VIP_1 and VIP_2 receptors mediate the activation of PLC, in addition to AC, when transiently expressed in COS 7 cells. This mechanism was found to be at least partially dependent on a PTx-sensitive G protein. The use of chimaeric receptors allowed the identification of a VIP_2 receptor domain likely to interact with the PTx-sensitive G protein. The portion of the receptor from the exchange site in TM5 to the exchange site in TM7 contains the relevant functional domain, which is likely to be the IC3 region of the receptor (the carboxyl terminal tail of the VIP_2 receptor was found to

have no identifiable effect on its signalling capability in terms of AC or PLC activation). The PACAP receptor was shown to mediate PLC stimulation by a different mechanism, which does not include PTx-sensitive G proteins. The GH_3 rat anterior pituitary tumour cell line (Tashjian, 1979) expresses VIP_2 receptors (E.M. Lutz; personal communication) and in these cells it was confirmed that native VIP_2 receptors, even when expressed at a low level, will mediate the stimulation of PLC. This demonstrates that PLC activation mediated by the VIP_2 receptor does not only occur as a result of promiscuous coupling of G proteins to the VIP_2 receptor, a phenomenon which has been attributed to high receptor expression level in a heterologous expression system (Zhu et al., 1994).

There are a number of instances in which VIP receptors have been found to act through PTx-sensitive G proteins, Diehl & Shreeve (1995) demonstrated an interaction between the VIP_2 receptor and $G\alpha_{i3}$ in rat lung and Murthy & Makhlouf (1994) found that VIP receptor-mediated effects in gastric smooth muscle cells were dependent on $G\alpha_{i1/2}$. In order to determine which PTx-sensitive G protein was involved in VIP receptor mediated stimulation of PI hydrolysis in COS 7 cells the HA-tagged human VIP_2 receptor was also transiently expressed in COS 7 cells and immunoprecipitated from membrane preparations of the cells to allow identification of the receptor-associated G proteins. In these experiments, Western blotting of the immunoprecipitate with specific anti-sera revealed the presence of $G\alpha_q$ and a member of the $G\alpha_{i/o/t/z}$ family, other than $G\alpha_{i1/2}$, associated with the human VIP_2 receptor. This suggests that the PTx-insensitive component of the VIP_2 receptor-mediated stimulation of PLC may be due to an interaction with G_q and the PTx-sensitive component is due to an interaction with a PTx-sensitive G protein other than $G\alpha_{i1/2}$.

Experiments on the rat VIP_2 receptor-mediated activation of PLC in COS 7 cells have revealed a role for Ca^{2+} influx through a plasma membrane channel sensitive to SK&F 96365, a calcium-release activated calcium influx ($CRAC_i$) and L-type Ca^{2+} channel inhibitor (Merritt et al., 1990). The concentration-dependent inhibition of VIP_2 receptor-mediated [^{3}H]IP production by Co^{2+} indicated a requirement for Ca^{2+} influx. The lack of effect of the classical L-type Ca^{2+} channel inhibitors, methoxyverapamil and nifedipine (Nowycky et al., 1985; Fox et al., 1987), on VIP_2 receptor-mediated [^{3}H]IP production, and the inhibitory effect of SK&F 96365, indicates that a Ca^{2+} channel other than an L-type Ca^{2+} channel is involved and that the mechanism may well involve a Ca^{2+} channel activated by the emptying of intracellular Ca^{2+} stores. Thapsigargin is a high affinity inhibitor of sarcoplasmic and endoplasmic reticulum Ca^{2+}-ATPases (SERCA: Thastrup et al., 1990). Treatment of cells with thapsigargin causes the emptying of the intracellular Ca^{2+} stores by inhibiting their ATP-dependent refilling mechanism and can thereby stimulate $CRAC_i$. Thapsigargin treatment proved to both stimulate [^{3}H]IP production and potentiate VIP_2 receptor-mediated [^{3}H]IP production providing further support for the idea of $CRAC_i$ being a fundamental part of this mechanism. The additional experiments which must be done to provide enough data to construct a detailed model of the system are outlined in Section 3.4. The simplest model that could currently be suggested involves the stimulation of PLC activity by both G_q and a PTx-sensitive G protein, the resultant production of inositol 1,4,5-trisphosphate causes the emptying of intracellular Ca^{2+} stores which in turn causes Ca^{2+} influx and the further stimulation of PLC activity. It is possible that a number of PLC isozymes are stimulated and that PTx-sensitive G proteins are involved at several stages of the process.

Murthy & Makhlouf (1995) also defined a role for Ca^{2+} influx in VIP-evoked relaxation of gastric smooth muscle. It is probable that the regulation of smooth

muscle relaxation is the major physiological role for VIP receptors. Gastric smooth muscle (Grider & Rivier, 1990; Gu et al., 1992; Murthy & Makhlouf, 1995), intestinal smooth muscle (Grider & Makhlouf, 1986), sphinteric smooth muscle (Goyal et al., 1995), vascular smooth muscle (Edvinsson et al., 1981) and airway smooth muscle (Foda et al., 1995) have all been shown to be responsive to VIP. The VIP receptors that we have demonstrated to be present in rat cerebral microvessels are likely to be expressed in the vascular smooth muscle cells. Murthy & Makhlouf (1995) described the VIP receptor-mediated relaxation of gastric smooth muscle as being due to both cAMP production and NO-evoked cGMP production. In this study, in GH_3 cells, it was demonstrated that the VIP_2 receptor-mediated the activation of AC, PLC and NOS.

It is not surprising that VIP receptor-mediated control of smooth muscle has proven to be the major focus of therapies based on modulating VIP receptor function. The human lung has been shown to contain VIP immunoreactive neurons (Carstairs & Barnes, 1986) and high levels of VIP (Polak & Bloom, 1982). Usdin et al. (1994) found VIP_1 receptor mRNA in large and moderate size bronchi and VIP_2 receptor mRNA in the smaller and terminal bronchioles. VIP_1 and VIP_2 receptor mRNA were both detected in the vascular epithelium of the lung. Ollerenshaw et al. (1989) suggest that the absence of VIP in the lung is responsible for the development of asthma since imunohistochemical methods failed to detect VIP in samples from asthmatic patients. Treatments with inhaled VIP however have proved ineffective (Altiere et al., 1984; Barnes & Dixon, 1984; Bundgaard et al., 1983; Mojarad et al., 1985). O'Donnell et al. (1994a;1994b) therefore designed a VIP analogue which was a more potent bronchodilator, and more resistant to proteolytic degradation, than VIP. This compound, Ro 25-1553, has also proven to be a more effective bronchodilator than isoproterenol or salbutamol and was found to be effective in relaxing airway smooth muscle and reducing inflammation in guinea pig lung *in vitro*

and *in vivo*. Ro 25-1553 was found to be highly selective for the VIP_2 receptor over the VIP_1 receptor (Gourlet et al., 1997). Both the VIP-evoked calcium influx mediated by the VIP_2 receptor in COS 7 cells and the activation of NOS in GH_3 cells that we have described are relevant to the observed relaxation of smooth muscle in lung since both nitric oxide and cAMP are known to facilitate relaxation of smooth muscle and NOS is likely to be activated by the influx of calcium as described by Murthy & Makhlouf (1995).

Similar mechanisms are likely to be present in the penis; VIPergic neurones innervate the cavernous smooth muscle and blood vessels. VIP levels are elevated during erections (Dixson et a., 1984; Ottesen et al., 1984) and Gu et al. (1984) found penile VIP to be reduced in impotent men. It was also observed that VIP can stimulate sexual behaviours in rats and that these effects can be reversed by a VIP/neurotensin hybrid peptide which acts as an antagonist of VIP (Gozes et al., 1989). Gozes et al. (1994) tested a VIP analogue, stearyl-norleucine-vasoactive intestinal peptide, as a treatment for impotence. This lipophilic VIP analogue was applied as an ointment, as opposed to by injection (which is more common for impotence treatments) and was found to be incorporated more effectively than VIP. The replacement of the methionine in position 17 of the VIP sequence with norleucine in stearyl-norleucine-vasoactive intestinal peptide increased its activity over that observed for VIP, presumably by reducing the sensitivity of the molecule to proteolytic degradation.

There is considerable evidence for VIP receptor-mediated control of the cerebral vasculature, not least the identification of VIP-releasing neurons innervating blood vessels in the rat cerebral cortex (Chedotal et al., 1994). In this study, RT-PCR of the RNA extracted from a rat brain microvessel preparation demonstrated the presence of VIP_1, VIP_2 and a low level of PACAP receptor mRNA. The

additional demonstration of VIP_2 receptor-mediated activation of NOS further strengthens the case for the involvement of the VIP_2 receptor in the control of the vasculature. Because of the role of VIP in regulating cerebral blood flow (Edvinsson et al., 1981; Anzai et al., 1995) it is possible that a VIP receptor agonist could be used to reduce ischaemic damage in stroke victims. Distinct from effects on blood flow, Brenneman et al. (1990) described a VIP-evoked increase in neuronal survival in dissociated spinal cord cultures suggesting a neuroprotective role for VIP receptor agonists.

VIP was also found to be an astroglial mitogen (Brenneman et al., 1990) and a growth factor for whole cultured mouse embryos (Gressens et al., 1993). However Maruno & Said (1993) found the growth of certain cell lines of small cell lung cancer to be inhibited by VIP treatment. VIP receptors have been found to be present in cells from breast, pancreatic and intestinal tumours (Waschek et al., 1995) and the possibility of regulating tumour growth through VIP receptor activity is being .investigated.

The VIP-evoked stimulation of melatonin secretion from the pineal gland has been well documented and is central to the photic regulation of circadian rhythms (Ibata et al., 1989; Watts & Swanson, 1990; Kalsbeek et al., 1992; Spessert, 1993; Simmoneaux et al., 1993; Duncan et al., 1995; Simmoneaux et al., 1998). It is possible that VIP receptor agonists may therefore be used to treat "jet-lag" and sleep disorders. In addition the close relationship between circadian rhythms and fertility in many mammals (McArthur et al., 1997; Duncan et al., 1995) suggests that VIP receptor agonists may provide a means of regulating fertility.

As a result of the large number of studies carried out to investigate the physiological roles of VIP and PACAP there are several promising therapeutic applications for VIP receptor agonists and antagonists. There are however many

questions still remaining regarding the fundamental intracellular signalling capabilities of the VIP receptors. This study has begun to address these issues.

Fahrenkrug (1993) suggested that the role of VIP in the brain is to regulate coupling between energy metabolism, blood flow and neuronal activity. Whereas Usdin et al. (1994) differentiate between the functions of the receptors in the brain suggesting that the VIP_1 receptor in the brain is primarily involved in regulating behaviour and higher cortical functions, because of it's presence in the cortex, hippocampus and amygdala, whereas the VIP_2 receptor is involved in sensory information processing, because of its presence in the brain stem, motor nuclei and thalamus. VIP also has activity in both the central and peripheral nervous systems and has been shown to be an important regulator of the immune (Ottaway, 1991) and endocrine systems (Koves et al., 1991; Rostene, 1984; Dow et al., 1994). It is apparent that a fuller understanding of the basic signalling characteristics of the VIP receptors will provide an invaluable insight into the regulation of a number of major physiological systems.

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Publications

Phospholipase C Activation by VIP_1 and VIP_2 Receptors Expressed in COS 7 Cells Involves a Pertussis Toxin-Sensitive Mechanism

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INTRODUCTION

The vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) receptors belong to the secretin/calcitonin/parathyroid hormone receptor family. This is a family of seven-transmembrane spanning G-protein-coupled receptors distinct from the rhodopsin and metabotropic glutamate receptor families.[1] Although all the known members of this family couple to adenylyl cyclase and a number have been shown to couple to phospholipase C (PLC),[2-6] their ability to couple to other second-messenger pathways remains largely to be elucidated. When expressed in COS 7 cells the VIP_1 and VIP_2 receptors efficiently activate adenylyl cyclase (AC)[7] and also phospholipase D (PLD) (D.A. McCulloch, unpublished data). VIP and PACAP have also been shown to stimulate nitric oxide synthase (NOS) in gastric smooth muscle cells through a common receptor and a mechanism involving a pertussis toxin (PTx)-sensitive G-protein.[8] We therefore investigated VIP_1 and VIP_2 receptor coupling to PLC and PLD and the potential PTx-sensitivity of these responses. In this paper we demonstrate PTx-sensitive coupling of the VIP_1 and VIP_2 receptors to PLC.

MATERIALS AND METHODS

COS 7 cells were transfected with cDNAs for the rat VIP_1, VIP_2, or PACAP receptors using DEAE dextran as previously described by Morrow and coworkers.[9] Twenty-four hours after transfection the cells were trypsinized and plated into 12 well plates. The VIP_1 cDNA (PV19) was a kind gift from Prof. S. Nagata.

[^{3}H]inositol Phosphates Assay

Cells in 12-well tissue-culture plates (maintained at 37°C in a 5% CO_2/95% O_2 environment) were labeled with 1 μCi/mL of *myo*-[2-^{3}H]inositol (Amersham) for 16

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h in Earle's Balanced Salt Solution with 10 mM glucose. At 48 h after transfection the cells were washed twice in Earle's Balanced Salt Solution with 10 mM glucose and 0.2% bovine serum albumin and preincubated for 10 min with 10 mM LiCl before agonist stimulation for 30 min. Reactions were stopped by aspiration of medium and addition of 700 μL of ice-cold 1.34-M trichloroacetic acid. The wells were scraped and the solution centrifuged to pellet the precipitated protein (5 min, 12,000 g). A 500-μL sample of the supernatant was then added to 50 μL of 0.1 M EDTA and 500 μL of a 1:1 mixture of 1,1,2-trichlorotrifluoroethane and tri-*n*-octylamine. The sample was vortexed and centrifuged (5 min, 12,000 g) and 300 μL of the aqueous phase was added to 200 μL of 1 M $NaHCO_3$ containing universal indicator. The sample was applied to a 1-mL column of Dowex anion exchange resin (1 × 8 resin, formate form, 200–400 mesh; Bio-Rad), and a stepwise gradient of ammonium formate was used to elute the [^{3}H]inositol phosphates, a method previously described by Berridge *et al.*[10] A 500-μL sample of the eluate was taken for scintillation counting.

Assay of Phospholipase D Activity

The phosphatidyl moiety of phosphatidylcholine was labeled by incubating the cells overnight with [^{3}H] palmitate (5 μCi per 0.5 mL at 37°C) under 5% CO_2/95% O_2. The cells were washed twice with warm (37°C) MEM containing 1% bovine serum albumin (BSA) fraction V. This was replaced with 1 mL warm MEM containing 0.5% BSA. Cells were incubated in the presence of 30 mM butan-1-ol to allow generation of [^{3}H] phosphatidylbutanol and the quantification of PLD activity. Reactions were terminated by aspiration of the assay medium and addition of 0.5 mL of ice-cold methanol. The 12 well plates were kept on ice and the cells scraped and transferred into 2-mL glass screw-top vials. Phosphatidylbutanol was extracted using the chloroform/methanol procedure as described previously by Bligh and Dyer.[11] The organic phase was retained and dried under vacuum in a centrifugal evaporator for 50 min at 30°C and resuspended in 50 μL chloroform/methanol (19:1). The sample was spotted onto a thin-layer chromatography silica gel plate and developed using the organic phase of a mixture of ethyl acetate/ 2,2,4-trimethylpentane/acetic acid/ water (110:50:20:100). The region of the plate corresponding to the running position of authentic phosphatidylbutanol was scraped and the radioactivity quantified by liquid scintillation counting.

When required, cells were preincubated for 16 hours with either 100 or 200 ng/mL pertussis holotoxin or its B subunit.

RESULTS

VIP (1 nM–3 μM) caused a concentration-dependent increase in [^{3}H]inositol phosphate formation with a 2.55 ± 0.16-fold increase over basal for the VIP_1 receptor and 1.99 ± 0.05-fold increase over basal for the VIP_2 receptor at a concentration of 3 μM. Treatment of the cells with 10 μM $AlCl_3$ and 50 mM NaF produced a 4.00

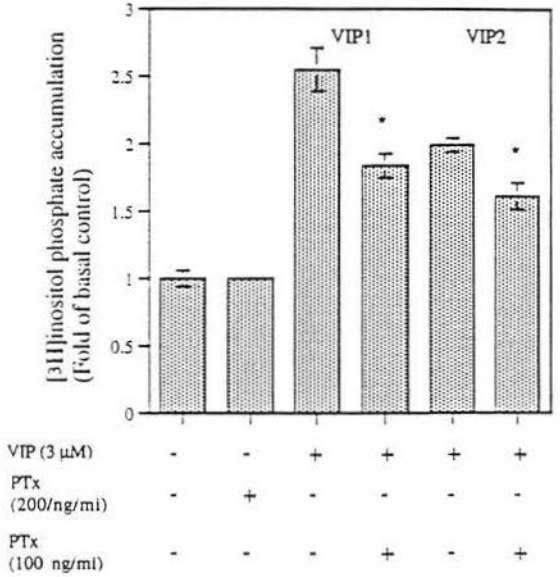

FIGURE 1. Pertussis toxin sensitivity of the VIP_1 and VIP_2 receptor-mediated PLC response. A typical basal activity was 10314 (±280) dpm/assay. Values are means ± S.E.M. from 6 separate determinations. There was no difference in basal [^{3}H]inositol phosphate formation in VIP_1 or VIP_2 receptor-expressing cells and data for these are expressed in a combined form. (*) Represents statistically significant inhibition of VIP-induced [^{3}H]inositol phosphate production ($p < 0.05$ by Mann-Whitney *U*-test).

± 0.57-fold increase over basal activity. FIGURE 1 demonstrates that PTx causes partial inhibition of [^{3}H]inositol phosphate production stimulated through the VIP_1 and VIP_2 receptors. PTx at 100 ng/mL reduced the VIP-stimulated response by 46 ± 9% for the VIP_1 receptor and 38 ± 10% for the VIP_2 receptor. A PTx treatment of 200 ng/mL had no effect on basal PLC activity (100 ± 2% of control). The addition of the PTx B subunit (200 ng/mL) had no significant effect on the VIP-stimulated PLC activity; the VIP_1 receptor maintained 100 ± 3% of its VIP stimulated activity and the VIP_2 receptor 99 ± 7%.

In order to determine whether a pathway mediated by the G-protein G_s could be responsible for either the PTX-sensitive or -insensitive components of PLC activation the cells were preincubated with cholera toxin (CTx). CTx caused no increase in basal [^{3}H]inositol phosphate formation (data not shown) and preincubation with 20 μg/mL CTx for 16 h (a protocol reported to downregulate G_s) had no effect on PLC responses to VIP. FIGURE 2 shows that in the presence of 20 μg/mL CTx the VIP-stimulated PLC response for the VIP_1 receptor was 105 ± 6% of control and for the VIP_2 receptor, 95 ± 6%.

Both VIP_1 and VIP_2 receptors showed concentration-dependent activation of PLD (100 nM-1 μM). One μM VIP elicited a 1.93 ± 0.13-fold over basal increase in [^{3}H]phosphatidylbutanol production through the VIP_1 receptor and 1.62 ± 0.24-fold

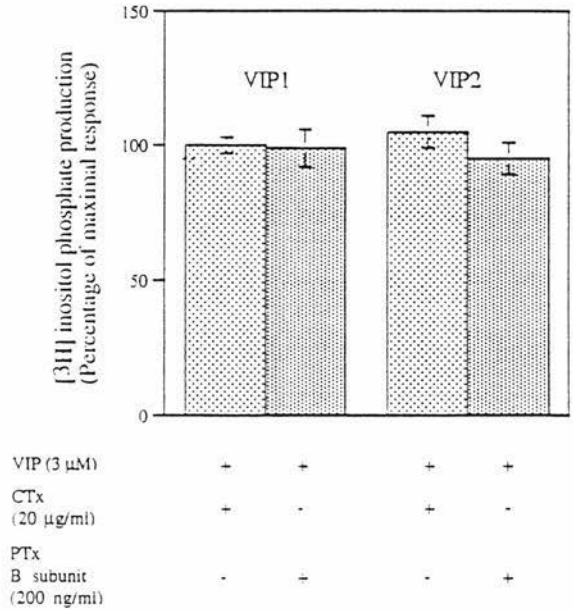

FIGURE 2. The effect of CTx and PTx B subunit on inositol phosphate production elicited by the VIP_1 and VIP_2 receptors. The control response to 3-μM VIP was typically around 29,869 dpm per assay. Toxins were present for 16 h prior to stimulation.

increase through the VIP_2 receptor. After PTx pretreatment the responses were 97 ± 4% and 109 ± 6% of the controls, respectively, for the VIP_1 and VIP_2 receptors.

FIGURE 3 shows the PTx-insensitivity of PLC activity stimulated through two splice variants of the PACAP type I receptor, the "short" and "long" forms. The long form includes a 28-amino-acid insert in intracellular loop III, the "hop1" cassette of Spengler *et al.*[5] One hundred nM PACAP-38 caused a 7.71 ± 0.55-fold increase over basal PLC activity for the short form of the receptor; in the presence of 200 ng/mL PTx this was a 7.81 ± 0.20-fold increase. The long form of the receptor mediated a 10.59 ± 0.82-fold increase over basal PLC activity in response to 100 nM PACAP-38; after PTx treatment the response was a 11.00 ± 0.27-fold increase over basal. PTx treatment alone had no effect on basal activity (91 ± 9% of control).

DISCUSSION

The present results demonstrate for the first time that VIP_1 and VIP_2 but not PACAP receptors can elicit a PTx-sensitive activation of PLC in a heterologous expression system. The stimulation of PLC through a PTx-sensitive G-protein is, however, well documented for a number of receptors of both rhodopsin and metabotropic glutamate receptor families.

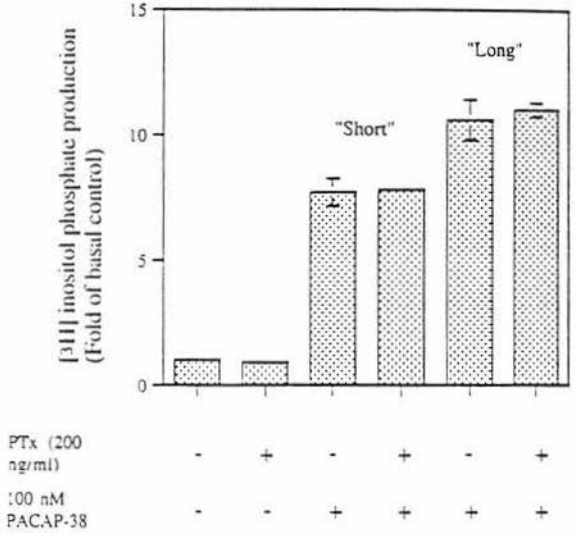

FIGURE 3. Pertussis toxin insensitivity of the PLC response mediated by 2 splice variants of the PACAP receptor. Values are means ± S.E.M. from 6 separate determinations.

The PLCβ1, β2, and β3 subtypes can all be activated by βγ subunits,[12] but only PLCβ1 and PLCβ3 were stimulated to any great extent by α subunits, particularly of the PTx-insensitive $G_{q/11}$ subfamily.[13] The fact that PTx treatment does not totally inhibit the VIP receptor-mediated PLC activity indicates that a portion of the response may be attributable to the more conventional $G_{q/11}$-mediated mechanism, whereas stimulation of PLC by G_i-derived by βγ subunits could account for the remainder.

These results suggest that in addition to G_s, VIP_1 and VIP_2 receptors are capable of interaction independently with G proteins of the $G_{q/11}$ and $G_{i/o}$ classes and that the ability to elicit $G_{i/o}$-mediated PLC activation is not shared by PACAP receptors.

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Chimaeric VIP_2/PACAP Receptors Reveal That Agonist Pharmacology but not Signal Transduction Is Determined by Extracellular Domain 1

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INTRODUCTION

Receptors for the secretin/glucagon family of large peptide hormones, which includes VIP and PACAP, belong to a family of seven-transmembrane spanning G-protein-coupled receptors that are distinct from the rhodopsin superfamily.[1] All members of this family couple to stimulation of adenylate cyclase, most probably mediated by G_s. Many are also capable of stimulating inositol phosphate production.[2-6] This receptor family has common structural features such as eight conserved cysteines, six of which are within a large N-terminal extracellular domain.

We have isolated cDNAs encoding two members of this family, the VIP_2 receptor[7] and two splice variants of the PACAP receptor, a long form containing an additional 84 bp cassette in the region encoding the third intracellular loop, and a short form which is without this cassette.[3] Structurally the short form of the PACAP receptor and the VIP_2 receptor are 50% identical at the amino acid level, the greatest similarity being within the transmembrane spanning regions. The PACAP receptors, when expressed in COS 7 cells, exhibit a distinctive pharmacological profile in that PACAP-38 is 100-fold more potent than VIP in eliciting cAMP stimulation.[3] In contrast, the VIP_2 receptor does not discriminate between the two peptides.[7] We have begun to characterize the domain responsible for agonist binding by domain swap experiments between the VIP_2 and PACAP receptors. Here we have replaced the 152-amino-acid extracellular domain of both the long and short forms of the PACAP receptor with the 125-amino-acid extracellular domain of the VIP_2 receptor. When these chimaeric receptors were expressed in COS 7 cells, we found that this exchange increases the receptor affinity for VIP such that it displays VIP_2-like pharmacology with PACAP-like coupling where both VIP and PACAP-38 are equipotent in the nanomolar range

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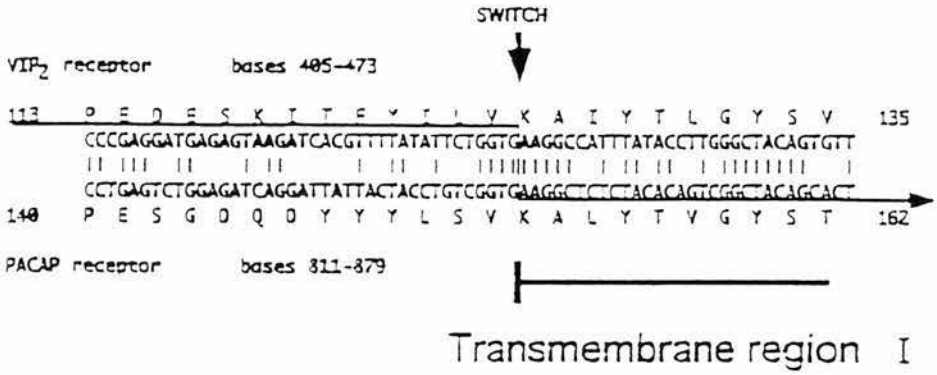

FIGURE 1. Lineup of VIP$_2$ receptor and PACAP receptor sequences spanning the boundary between the extracellular I domain and transmembrane region I domain. The *arrow* marks the spot at which the crossover by primer M3347 is made.

at stimulating cAMP and inositol phosphate production. This suggests that the amino terminal domain of the VIP$_2$ receptor is sufficient for determining the pharmacological specificity of agonist binding.

MATERIALS AND METHODS

Preparation of Chimaeric cDNA

In order to make the chimaeric receptors, N4/9.1 and N4/7b, two oligonucleotide primers (Oswel DNA Service, Department of Chemistry, University of Edinburgh, Scotland) were designed to the VIP$_2$ receptor (R4, pBluescript) and the PACAP receptor encoding cDNAs (R7 and R7b, pBluescript). In the first round of PCR amplification, the primer, 1143 (5′CACCAGAATATAAAACGTGATCTTAC) along with pBluescript primer, was used to amplify the 5′ region of the R4 cDNA. The primer, M3347, (5′CGTTTTATATTCTGGTGAAGGCTCTCTACACAGTC) was designed to overlap the cDNAs encoding the region of the extracellular domain I/ transmembrane domain I of the VIP$_2$ and PACAP receptors (see FIG. 1). Along with the pBluescript primer, M3347 was used to amplify the 3′ region of both the long and short forms of PACAP receptor encoding cDNAs. For the second round of PCR amplification, the 5′ R4 PCR fragment and either the M3347 primed R7 or R7b PCR fragments were mixed and amplified using pBluescript primers. The PCR products were digested with Eco RI and ligated into pBluescript for sequence analysis. After selection of a positive clone for each, a further step involved ligation of the 2.14-kb and 2.06-kb Eco RI fragments into the expression vector pCDNA (Invitrogen, R& D Systems Europe Ltd., Abingdon, UK) and selection of clones in the proper orientation (N4/9.1 and N4/7b).

Experimental

COS 7 cells were transfected using DEAE dextran as described previously[3] and allowed to recover for 24 h before trypsinization and replating. For measurement of

inositol phosphate production. 24 h after trypsinization, cells (in 12 well plates) were labeled with [^{3}H]-myo-inositol (1 μCi/mL) for 16 h, washed, then incubated in medium containing 10 mM LiCl for 15 min before challenging with various peptides for 30 min. Reactions were stopped in ice-cold trichloroacetic acid, cells scraped from the wells and centrifuged before extracting the supernatant in an equal volume (50 : 50 mix) 1,1,2-trichlorotrifluoroethane/tri-*n*-octylamine. The aqueous phase was neutralized to pH 7-8 with $NaHCO_3$. Inositol phosphate were separated by ion exchange on Dowex AG 1-X8.[9] The production of cAMP by cells (in 24 well plates) was measured during a 20-min stimulation period as described previously.[3]

RESULTS AND DISCUSSION

The PACAP receptor is able to couple to both adenylate cyclase and phospholipase C in transfected COS 7 cells when stimulated by PACAP-38,[10] with EC_{50} values of 0.5 nM and 7 nM, respectively, for each pathway.[3,10] VIP is approximately 100-fold less potent in stimulating cAMP production at this receptor.[3] The VIP_2 receptor does not appear to discriminate between VIP or PACAP: both are equivalent in stimulating cAMP production in COS 7 cells transfected with this receptor's cDNA, with EC_{50}'s of 0.17 and 0.18 nM, respectively.[7] We have characterized two chimaeric receptors where we replaced the amino-terminal region of the PACAP receptor with that of the VIP_2 receptor, at the approximate boundary between extracellular domain I and the first transmembrane region. Oligonucleotide primers to the region encoding the junction between extracellular domain/transmembrane region one were designed for both the PACAP and the VIP_2 receptor cDNAs to enable the crossover during PCR amplification, to create cDNAs encoding receptors with the 125-amino-acid N terminal of the VIP_2 receptor followed by the 343-amino-acid (long form) or 259-amino-acid (short form) C terminal of the PACAP receptor. When expressed in COS 7 cells, the chimaeric receptors (N4/9.1 and N4/7b, respectively) closely resemble the wild-type VIP_2 receptor in their ability similarly to couple to adenylate cyclase when stimulated with PACAP-38 or with VIP (see FIG. 2(a)). In addition, these chimaeric receptors activated phospholipase C when stimulated with either peptide (FIG. 2(b)), in a manner similar to the PACAP receptor when activated by PACAP-38 (data not shown[10]). The presence of the additional 28-amino-acid insert in intracellular loop three (long form) did not appear to affect the functional aspects of the chimaeric receptors that were assessed here. TABLE 1 shows the respective EC_{50} values for PACAP-38 and VIP at these two receptors. This suggests that the first extracellular domain of the VIP_2 receptor is capable of determining the agonist pharmacology of these chimaeric receptors, but that the signal transduction capabilities are conferred by the remainder of the molecule.

The large extracellular domain I of the secretin/PTH/calcitonin receptor family contains several conserved amino acids, including six cysteines. Mutation of a conserved aspartate (residue 60), in the mouse GHRH receptor gene disrupts GHRH interaction with its receptor and has been identified as the cause of the "Dwarf" *little* mouse syndrome.[11] Several conserved residues within the N-terminal domain of the human VIP_1 receptor are important for VIP binding.[12] A clone of the human VIP_1 receptor that contains an alternative 67-amino-acid N-terminal region combined

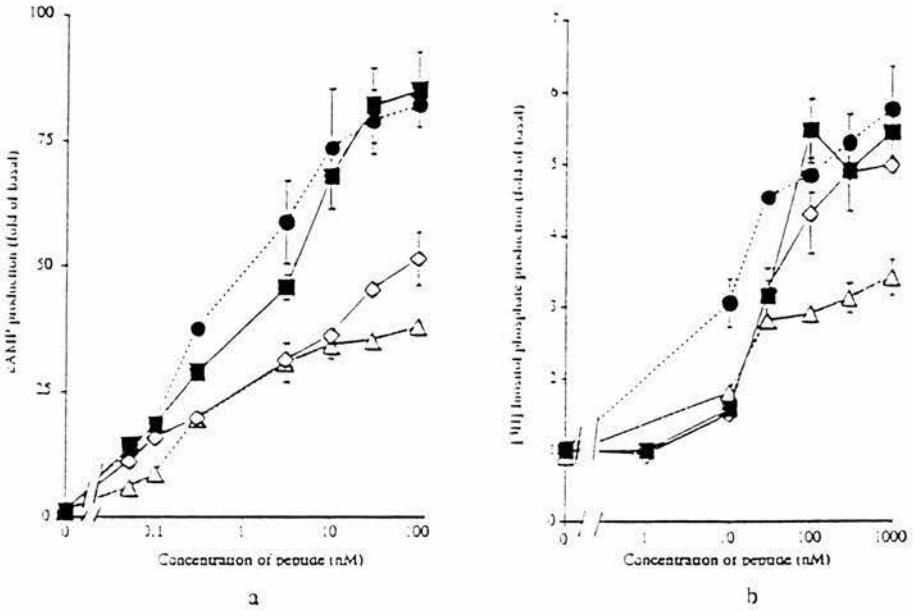

FIGURE 2. COS 7 cells transiently transfected with the VIP$_1$(1-125)/PACAP(153-495) chimaeric receptors (encoded by N4/9.1, which contains the extra 84-bp exon in the region encoding intracellular loop 3, and N4/7b, which does not contain this exon) were stimulated with VIP (■ N4/9.1; ◇ N4/7b) and PACAP-38 (● N4/9.1 △ N4/7b). The values represent the mean (± SEM, n = 3-5). (a) cAMP levels in response to a 20-min stimulation with each peptide were measured. Basal levels were 2 ± 1 pmol/well. (b) [^{3}H]inositol phosphate levels in response to a 30-min stimulation with each peptide were measured. Basal levels were 3387 ± 669 dpm per well. Error bars not shown fall within the dimension of the symbol.

TABLE 1. Table of EC_{50} Values for PACAP-38 and VIP in COS 7 Cells Transfected with the Chimaeric Receptors N4/9.1 (VIP$_1$1-125/PACAP153-495, long form) and N4/7b (VIP$_1$1-125/PACAP153-495, short form)[a]

Expressed Receptor	Treatment	EC_{50} cAMP Production	EC_{50} Inositol Phosphate Production
N4/9.1	PACAP-38	0.5 ± 0.1 nM	9.5 ± 0.8 nM
	VIP	1.1 ± 0.1 nM	36.5 ± 9.8 nM
N4/7b	PACAP-38	0.5 ± 0.04 nM	10.8 ± 0.7 nM
	VIP	0.5 ± 0.03 nM	27.6 ± 1.6 nM

[a] Results are the means ± SEM from 3 to 5 experiments.

with the 428 residues of the C terminus is expressed normally but does not bind ^{125}I-VIP.[13] Replacing the N-terminal extracellular domain of the secretin receptor with the N-terminal extracellular domain of the rat VIP$_1$ receptor confers VIP pharmacology to the chimaeric receptor.[14] However, the chimaeric receptor that was created by the reverse transfer did not respond to secretin in a manner similar to its wild-type

receptor, and required the additional transfer of the second extracellular domain along with the first for this to be observed.[14] Residues near the N terminal and also within the third extracellular loop have been found to be important for PTH binding.[15]

In conclusion these results indicate that in the VIP_2 receptor agonist recognition appears to be achieved entirely within the first extracellular domain. While this appears to be the case in certain other members of the family (e.g., VIP_1 receptor[14]), other related receptors, for example, the secretin and the PTH receptor, clearly have more complex multisite requirements for agonist recognition.

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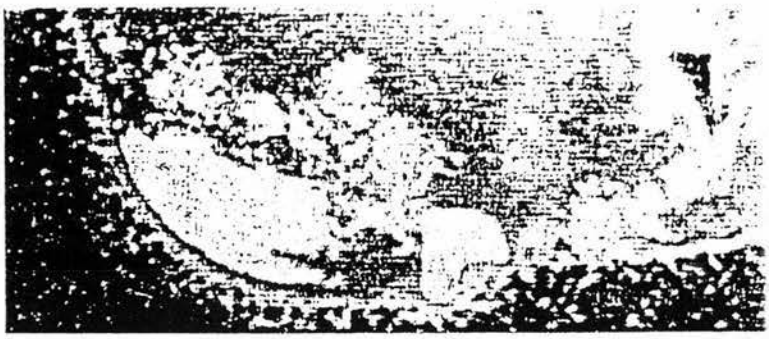

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ABSTRACTS ISSUE: ABSTRACTS FROM THE 11TH INTERNATIONAL SYMPOSIUM ON REGULATORY PEPTIDES

REGULATORY PEPTIDES

ELSEVIER

Endothelin and NPY receptors in human parathyroid gland.

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Endothelins and NPY are distributed in neuronal elements both within CNS and in the periphery, and endothelins also in non-neuronal cells. They are thought to play important roles as messengers. Endothelins and NPY are known to be vasoconstrictors in the periphery, but also non-vascular smooth muscle, and exocrine as well as endocrine glands, are known as targets. Endothelin-1 has been demonstrated in the pituitary,adrenal and parathyroid glands and has been shown to increase intracellular calcium in a bovine parathyroid endothelial cell line. In human parathyroid glands and in parathyroid glands of several other species NPY occurs in nerve fibers which predominate around blood vessels. In this study we used reverse transcriptase - polymerase chain reaction (RT-PCR) to demonstrate endothelin receptors, ET^A and ET^B, and the NPY receptor, Y_1, in human normal and hyperplastic parathyroid glands and in parathyroid adenomas. Since endothelin and NPY have been demonstrated in human parathyroid glands we were interested to know whether the receptor mRNAs were detectable and which receptors were dominating. We found ET^A-, ET^B- and Y_1-receptors in all tissue samples analyzed. ET^B-receptors were the predominating receptor in both normal glands and adenomas. ET^A and ET^B receptors are functionally coupled to phospholipase C-mediated phophoinositide hydrolysis and activation of these receptors brings about an increase in intracellular Ca^{2+} and Ca influx. Endothelin-1 may therefore induce receptor-mediated increase in intracellular Ca^{2+} concentrations to inhibit basal PTH secretion from parathyroid cells. The abundant expression of ET receptors in parathyroid tissue favors a role of endothelins as local modulators of PTH secretion. The Y_1 receptors are likely to be expressed in intraglandular blood vessels and involved in the regulation of local blood flow.

SIGNALLING BY THE WILD TYPE RAT VIP_2 RECEPTOR AND CARBOXYL TAIL TRUNCATED FORMS TRANSIENTLY EXPRESSED IN COS 7 CELLS

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Receptors for VIP and the VIP-like peptide PACAP belong to a distinct subgroup of the seven transmembrane-spanning G protein-coupled receptor family which includes receptors for secretin, PTH and calcitonin. All members of this family couple to adenylate cyclase, through the heterotrimeric G protein G_s and are capable of activating multiple signal transduction pathways. We and others have shown that the PACAP receptor couples to phospholipase C via a pertussis toxin-insensitive G protein, most likely G_q. In contrast, VIP_1 and VIP_2 receptors transiently expressed in COS 7 cells activate [^{3}H]inositol phosphate formation through a mechanism which is in part pertussis toxin-sensitive (MacKenzie *et al.*, Ann. NY Acad. Sci., in press). Here we report comparison of the wild type rat VIP_2 receptor (VIP_2^{wt}) and two carboxyl tail truncated forms of 391 amino acids, VIP_2(Δ392-437) and 376 amino acids, VIP_2(Δ377-437). COS 7 cells transfected with VIP_2(Δ377-437) yielded no detectable specific binding of [^{125}I]PACAP-27 or stimulation of cAMP production in response to VIP. The 391 amino acid VIP_2(Δ392-437) was well expressed and functional in COS 7 cells, showing levels of specific [^{125}I]PACAP-27 binding similar to VIP_2^{wt}. The Kd for VIP_2(Δ392-437) was however markedly lower than for VIP_2^{wt}. VIP_2(Δ392-437) elicited cAMP production in response to VIP and helodermin with potencies very similar to VIP_2^{wt}(Table 1).

Table 1 Table of EC_{50} values for cAMP stimulation by the VIP_2 and carboxyl tail truncated VIP_2(Δ392-437) receptor

Receptor	VIP	Helodermin
wild type	0.11 ± 0.01 nM	0.25 ± 0.005 nM
VIP_2(Δ392-437)	0.10 ± 0.01 nM	0.11 ± 0.01 nM

The initial rate of cAMP stimulation and its time dependent desensitisation were also similar for VIP_2^{wt} and VIP_2(Δ392-437) receptors. Pertussis toxin inhibited VIP and PACAP-38 evoked [^{3}H]inositol phosphate formation in cells expressing either VIP_2^{wt} or VIP_2(Δ392-437). However EC_{50}s for [^{3}H]inositol phosphate formation mediated by VIP_2^{wt} (31 ± 8 nM and 35 ± 5 nM for PACAP 38 and VIP respectively) were lower than that for VIP_2(Δ392-437) (64 ± 3 nM and 68 ± 15 nM respectively). Basal levels of cAMP and of [^{3}H]inositol phosphate were similar in cells transfected with VIP_2^{wt} or with the truncated receptors. The 46 amino acid truncation of the VIP_2 receptor therefore increases ligand affinity whilst affecting signalling through one pathway ([^{3}H]inositol phosphate) but not affecting another (cAMP). It is apparent from studies of truncated calcitonin and PTH receptors that the carboxyl tail is involved in normal protein trafficking and expression of the receptor. Effects on effector coupling were less clear cut. Coupling to cAMP stimulation was very similar in wild type and truncated receptors which were normally expressed. However the Ca^{2+} signal was affected by carboxyl tail truncation of the pig calcitonin receptor, whereas for human and for opposum PTH receptors it was unaffected by reduced carboxyl tail length. Further studies will be necessary to elucidate the mechanisms involved.

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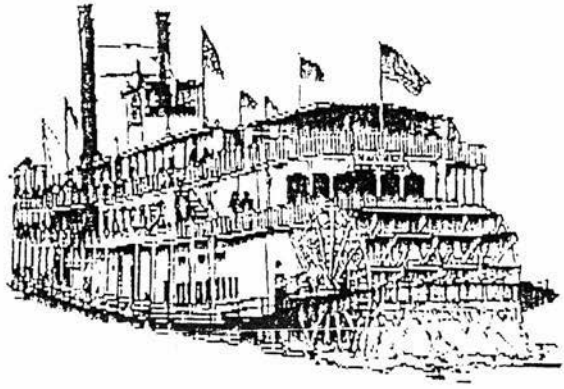
PROGRAM/ABSTRACTS

2ND INTERNATIONAL SYMPOSIUM ON VIP, PACAP, & RELATED PEPTIDES

OCTOBER 4-7, 1995



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2ND INTERNATIONAL SYMPOSIUM ON VIP, PACAP, & RELATED PEPTIDES, NEW ORLEANS USA, 4-7 OCTOBER 1995

CHIMERIC VIP_2/PACAP RECEPTORS REVEAL THAT AGONIST PHARMACOLOGY BUT NOT SIGNAL TRANSDUCTION IS DETERMINED BY EXTRACELLULAR DOMAIN 1

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Receptors for the secretin/glucagon family of large peptide hormones, which includes VIP and PACAP, belong to a family of seven transmembrane spanning G protein-coupled receptors which are distinct from the rhodopsin superfamily. All members of this family are coupled to stimulation of adenylate cyclase, most probably mediated by G_S. Many are also capable of stimulating inositol phosphate production. We have isolated cDNAs encoding two members of this family, the VIP_2 receptor and two splice variants of the PACAP receptor. When expressed in COS 7 cells, the short and long forms of the PACAP receptor couple in a very similar fashion to both stimulation of cAMP and inositol phosphate production. PACAP-38 is 100 fold more potent than VIP in eliciting cAMP production, with an EC_{50} of 0.5 nM. PACAP-38 stimulates inositol phosphate production with an EC_{50} of 7 nM whereas VIP was inactive at concentrations up to 1 μM. Both PACAP-38 and VIP are equipotent at the VIP_2 receptor in stimulating cAMP production, with EC_{50}s of 0.18 and 0.17 nM, respectively.

Structurally the unspliced PACAP receptor and the VIP_2 receptor are 50% identical at the amino acid level. We have designed oligonucleotides to a region at the extracellular domain/transmembrane region one junction for PCR amplification in order to exchange the amino terminal extracellular domains of the two receptors. Two chimeric receptors which we have created replace the N-terminal 152 amino acids of both the short and long forms of the PACAP receptor with the 125 amino acid N-terminal of the VIP_2 receptor. This exchange increases the receptor affinity for VIP such that it displays VIP_2-like pharmacology with PACAP-like coupling where both VIP and PACAP-38 are equipotent in the nanomolar range at stimulating cAMP and inositol phosphate production. This suggests that the amino terminal domain of the VIP_2 receptor is sufficient for determining the pharmacological specificity of agonist binding by these receptors.

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2ND INTERNATIONAL SYMPOSIUM ON VIP, PACAP, & RELATED PEPTIDES, NEW ORLEANS USA, 4-7 OCTOBER 1995

PHOSPHOLIPASE C ACTIVATION BY VIP_1 AND VIP_2 RECEPTORS EXPRESSED IN COS 7 CELLS INVOLVES A PERTUSSIS TOXIN-SENSITIVE MECHANISM

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The VIP_1 and VIP_2 receptors are members of the secretin/glucagon/parathyroid hormone family of G protein-linked seven transmembrane spanning receptors. The ability of the VIP_1 and VIP_2 receptors to interact with different G proteins and to activate different second-messenger pathways largely remains to be elucidated. When expressed in COS 7 cells the VIP_1 and VIP_2 receptors have been shown to couple to the adenylate cyclase, phospholipase C (PLC) and phospholipase D (PLD) pathways. VIP and PACAP-38 have also been shown to stimulate nitric oxide synthase in gastric smooth muscle cells through a common receptor and a pertussis toxin (PTx)-sensitive G protein.

We have demonstrated that activation of PLC mediated by the VIP_1 and VIP_2 receptors involves a Ptx-sensitive mechanism. Stimulation of the VIP_1 receptor with 3 μM VIP caused a 3.64 ± 0.18 -fold increase over basal [^{3}H]inositol phosphate production, the VIP_2 receptor a 1.96 ± 0.09 -fold increase, while 50 mM AlF_4^- elicited a 4.00 ± 0.57 -fold increase over basal. Pretreatment with 100 ng/ml PTx caused a 46% ± 10% inhibition of PLC activity stimulated through the VIP_1 receptor and a 38% ± 17% inhibition of VIP_2 receptor mediated activity. PTx treatments up to 200 ng/ml had no effect on basal PLC activity.

In contrast, the PLC response mediated by two splice variants of the PACAP receptor ("short" and "long" intracellular loop 3 sequences) was not PTx-sensitive. 100 nM PACAP-38 caused a 6.75 ± 0.05 -fold increase over basal [^{3}H]inositol phosphate production for the "short" receptor and 8.87 ± 0.44 -fold increase for the "long" receptor. In the presence of PTx the responses were 101% ± 6% and 104% ± 7% of control respectively for the "short" and "long" form receptors. VIP_1 and VIP_2 receptor-mediated PLD activity is also PTx-insensitive. 1 μM VIP elicited a 1.93 ± 0.13 -fold over basal increase in [^{3}H]phosphatidylbutanol production through the VIP_1 receptor and 1.62 ± 0.24 -fold increase through the VIP_2 receptor. After PTx pretreatment the responses were 97% ± 4% and 109% ± 6% of the controls respectively for the VIP_1 and VIP_2 receptors.

Rhodopsin-family receptors associate with small G proteins to activate phospholipase D

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G-protein-coupled receptors of the rhodopsin family transduce many important neural and endocrine signals. These receptors activate heterotrimeric G proteins and in many cases also cause activation of phospholipase D, an enzyme that can be controlled by the small G proteins ARF and RhoA[1–3]. Here we show that the activation of phospholipase D that is induced by many, but not all, Ca^{2+}-mobilizing G-protein-coupled receptors is sensitive to inhibitors of ARF and of RhoA. Receptors of this type were co-immunoprecipitated with ARF or RhoA on exposure to agonists, and the effects of GTP analogues on ligand binding to the receptor changed to a profile that is characteristic of small G proteins. These receptors contain the amino-acid sequence Asn-ProXXTyr in their seventh transmembrane domain, whereas receptors capable of activating phospholipase D without involving ARF contain the sequence AspProXXTyr. Mutation of this latter sequence to AsnProXXTyr in the gonadotropin-releasing hormone receptor conferred sensitivity to an inhibitor of ARF, and the reciprocal mutation in the 5-HT_{2A} receptor for 5-hydroxytryptamine reduced its sensitivity to the inhibitor. Receptors carrying the AsnProXXTyr motif thus seem to form functional complexes with ARF and RhoA.

The activation of phospholipase D (PLD) (measured as formation of [^{3}H]phosphatidylbutanol) by a number of receptors that are native to 1321N1 human astrocytoma cells[4] was differentially inhibited by brefeldin A (BFA), an inhibitor of guanine-nucleotide exchange on ARF[5]. Activation of PLD by H_1 histamine, B_2 bradykinin and M_3 muscarinic receptors in these cells[6] (as for M_3 receptors expressed in HEK 293 cells[7]) was sensitive to BFA whereas activation of PLD by thrombin and thromboxane A_2 receptors was BFA-resistant (Fig. 1a). Thrombin- but not M_3-receptor responses were attenuated by the phospholipase C (PLC) inhibitor U73122, but neither response was affected by pertussis toxin (Fig. 1b). The Rho inhibitor C3 exoenzyme and a negative construct, CMV5 Asn 19 RhoA (ref. 8), both reduced M_3- but not thrombin-receptor-mediated activation of PLD, whereas wild-type RhoA increased responses to a lower concentration of carbachol but not thrombin (Fig. 1b). These results indicate that some Ca^{2+}-mobilizing G-protein-coupled receptors (GPCRs) use a pathway of PLD activation that is independent of Gq/11 (which activates PLC) and Gi/o, yet involves ARF and RhoA.

We used co-immunoprecipitation to test whether M_3-receptor-mediated PLD activation might involve a step in which the receptor and small G proteins interact closely. Figure 1c shows that solubilized M_3 receptors (levels of which were measured by binding of [^{3}H]*N*-methyl scopolamine ([^{3}H]NMS)) could be immunoprecipitated using polyclonal antibodies against ARF1/3 (ref. 9) or RhoA[10]. Yields from using combinations of antibodies were less than additive. Co-immunoprecipitation required pre-exposure to agonists (priming) and there was little co-imunoprecipitation when

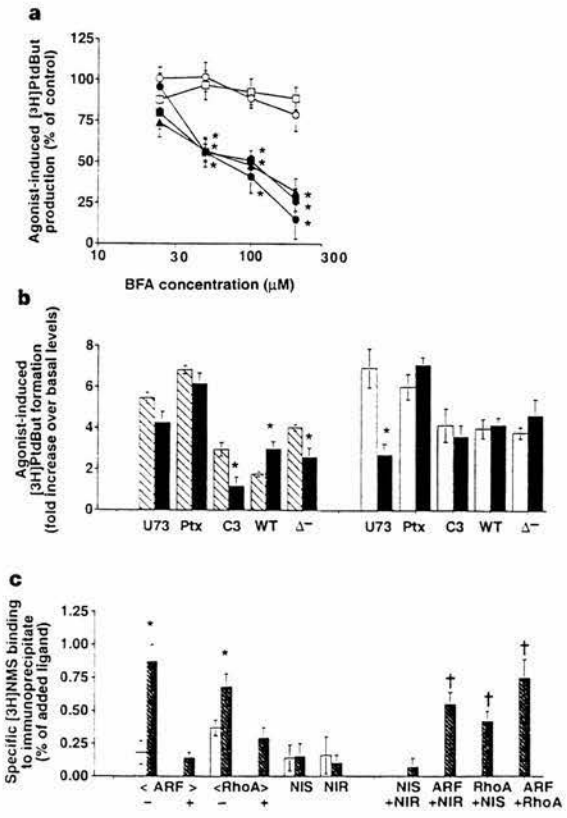

Figure 1 Properties of agonist-evoked PLD responses in 1321N1 cells and co-immunoprecipitation of M_3 receptors with ARF1/3 and RhoA antibodies. **a**, the effects of BFA on [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) production elicited by: ● 200 μM carbachol, ■ 10 μM bradykinin, ▲ 2 mM histamine, ○ 0.5 units per ml thrombin, or □ 30 μM U46619 (a TXA_2-receptor agonist). IC_{50} values for effects of BFA on M_3, bradykinin and histamine responses were 72 ± 11, 78 ± 15 and 82 ± 13 μM, respectively, whereas thrombin-receptor and TXA_2-receptor responses were resistant up to 200 μM BFA. **b**, Production of PtdBut in response to carbachol and thrombin is shown by hatched and open columns, respectively. Corresponding values for reagent-treated cells are shown by adjacent black columns. Reagents were U73122 (20 μM; U 73), pertussis toxin (200 ng ml^{-1} for 18 h; Ptx), C3 exoenzyme (4.8 μg ml^{-1}; C3) and wild-type or negative RhoA constructs (WT and Δ$^-$). Carbachol was used at a concentration of 200 μM except in C3 and WT RhoA experiments, where 100 μM and 20 μM, respectively, were used. Thrombin was usually 0.5 units per ml but 0.2 units per ml in WT RhoA experiments. In **a**, **b**, values are means ± s.e.m.; n = 4–10; significant changes from the control levels are indicated by asterisks ($P < 0.05$; Wilcoxon test). **c**, Solubilized membrane proteins from cells preincubated with carbachol (hatched columns) or from controls (open columns) were immunoprecipitated with sheep anti-ARF1/3 antibody (ARF), rabbit anti-RhoA antibody (RhoA), non-immune sheep IgG (NIS) or non-immune rabbit IgG (NIR) or combinations of these antibodies, before labelling M_3 receptors with [^{3}H]NMS. In some ARF/RhoA immunoprecipitations, blocking peptides were included (shown as ±). Values are means ± s.e.m. (n = 6–9). For asterisked bars, $P < 0.05$ compared with unprimed controls, and for bars with daggers $P < 0.05$ compared with non-immune IgG controls (Wilcoxon test). We estimate that up to 48% and 27% of ligand binding to solubilized M_3 receptor may be associated specifically (in a peptide-blockable manner) with ARF and RhoA immunoprecipitates after agonist priming.

using control immunoreagents or in the presence of excess peptide antigen. Use of BFA (100 μM) during priming reduced the yield of M_3 receptors in ARF1/3 immunoprecipitates by 74 ± 19%. Thrombin receptors, labelled with [^{125}I]Ala-pFPhe-Arg-Cha-HArg-Tyr-NH_2 ([^{125}I]TRP; ref. 11) could not be co-immunoprecipitated by antibodies against ARF/Rho after priming with thrombin agonist (data not shown). When a control immunoprecipitating antibody (anti-PKCα, Transduction Laboratories) was used to collect another signalling protein known to translocate to 1321N1 cell membranes, preincubation with carbachol caused no increase in [^{3}H]NMS binding to immunoprecipitates despite a 3.1 ± 0.3-fold increase in membrane binding of [^{3}H]phorbol 12,13-dibutyrate (data not shown).

Immunoblotting using distinct (monoclonal) antibodies showed that authentic ARF and Rho were present in ARF/Rho-directed immunoprecipitates from carbachol-primed cells; ARF and Rho were not present in immunoprecipitates when peptide antigens to the polyclonal reagents were added (Fig. 2a, b). In the detergent/NaCl conditions that we used, the monoclonal anti-ARF antibodies ID9 (ref. 12) and clone 26 (Transduction Laboratories) were much less effective or completely ineffective, respectively, at precipitating ARF immunoreactivity and [^{3}H]NMS-binding sites (data not shown). An M_3-receptor antiserum fraction[6], but not non-immune IgG, caused co-precipitation of immunoreactive ARF and RhoA after priming with carbachol (Fig. 2c, d). Similarly, polyclonal antibodies to the AT_1 receptor (BFA inhibited PLD activation by this receptor; half-maximal inhibitory concentration (IC_{50}) 58 ± 5 μM) caused priming-dependent, peptide-blockable co-precipitation of authentic ARF (Fig. 2e) and Rho (data not shown). In addition, when AT_{II}-primed membranes were treated with the crosslinker ethylene glycolbis(succinimidylsuccinate) and immunoprecipitations carried out with antibody ID9 in the presence of Triton X-100/SDS, specific AT_1-receptor immunoreactivity became detectable in immunoprecipitated high-molecular-mass complexes of approximately 170K (data not shown). These results indicate that certain receptors may be able to form physical complexes involving ARF1/3 and/or RhoA and that this may be important in the activation of PLD.

We used GTP analogues to determine whether the effects of these analogues on both receptor and effector properties were consistent with the above hypothesis. The affinity of M_3 receptors labelled with [^{3}H]NMS for carbachol was reduced by guanosine 5′-0-(3-thio)-triphosphate (GTPγS), BeF_3^- (an isostere of AlF_4^- (ref. 13)) and guanosine 5′-[βγ-methylene]triphosphate (GPPCH$_2$P) (Table 1a).

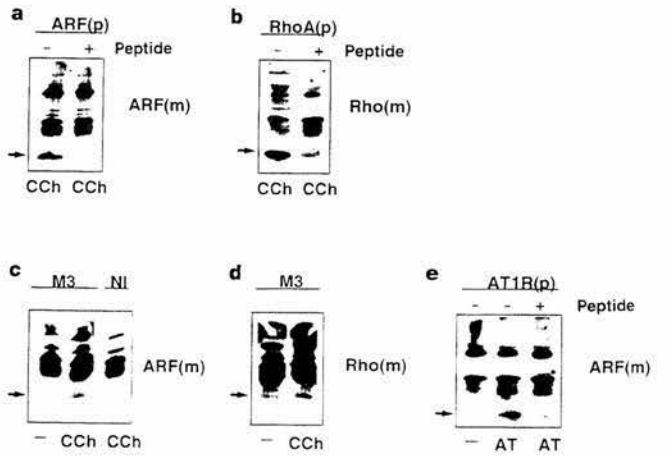

Figure 2 Immunoblots for ARF and Rho on immunoprecipitates generated with polyclonal ARF1/3, RhoA and receptor antibodies. Blots **a**–**d** are from 1321N1 cells and **e** is from rat anterior pituitary cells. For each panel, the labels below indicate the preincubation conditions (CCh, carbachol; AT, ATII; -, control. Labels above show the immunoprecipitation reagents (ARF(p), sheep polyclonal anti-ARF1/3 antibody; RhoA(p), rabbit polyclonal anti-RhoA antibody; M_3, rabbit polyclonal anti-M_3-receptor antibody; NI, non-immune rabbit IgG; AT1R(p), rabbit polyclonal anti-AT_1-receptor antibody), with (+) or without (–) blocking peptides. Labels to the right indicate the immunoblotting reagents (ARF(m), mouse monoclonal anti-ARF antibody; Rho(m), mouse monoclonal anti-Rho antibody). The arrow indicates the position of the 20 kDa molecular mass standard.

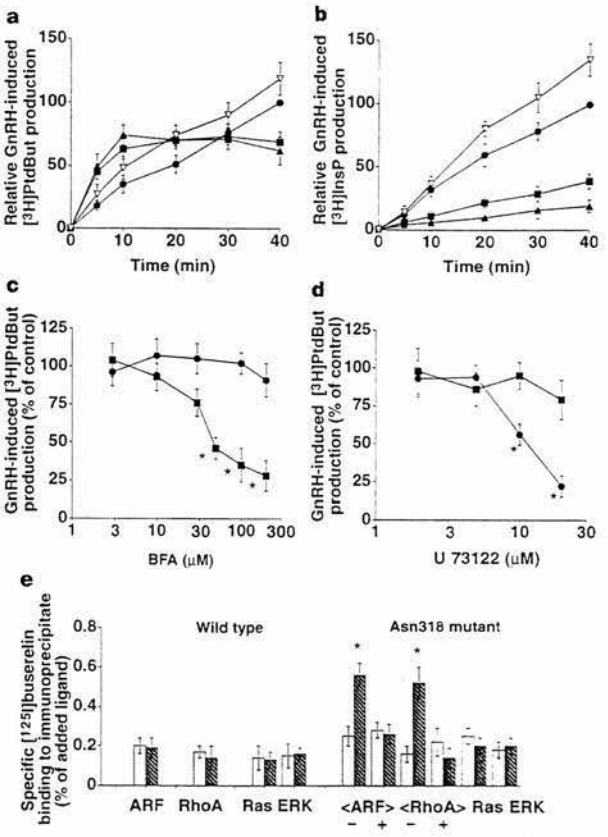

Figure 3 PLD and PLC responses of wild-type, Asn 318 and Asp 318 plus Asp 87 Asn 318 mutant GnRH receptors expressed in COS 7 cells and receptor immunoprecipitation with ARF1/3 and RhoA antibodies. **a**, **b** Show the time course of [^{3}H]phosphatidylbutanol ([^{3}H]PtdBut) and [^{3}H]inositol phosphate ([^{3}H]InsP) production evoked by 100 nM GnRH at ● wild-type, ■ Asn 318 mutant and ▲ Asp 87 Asn 318 mutant receptors and by ▼ AlF_4^- (10 mM NaF and 30 μM $AlCl_3$) in Asn 318 mutant expressing cells. Values are percentages of wild-type response at 40 min (means ± s.e.m.; n = 4–6). **c**, **d** Show effects of BFA and U73122 on [^{3}H]PtdBut production evoked by 100 nM GnRH at ● wild-type and ■ Asn 318 mutant receptors. Values are means ± s.e.m.; n = 4–6. **e**. Shows binding of [^{125}I]buserelin to immunoprecipitates from cells expressing wild-type or Asn 318 mutant receptors. Extracts from cells preincubated with GnRH (hatched columns) or from controls (open columns) were immunoprecipitated with antibodies against ARF1/3 (ARF), RhoA (RhoA), p21Ras (Ras) or ERK1/2 (ERK). Blocking peptides for ARF/RhoA antibodies were sometimes included (indicated as ±). Values are means ± s.e.m.; n = 4–8. Asterisks relate to P values of <0.05 compared to controls, by Wilcoxon test. After agonist priming, 26% and 33% of ligand binding to solubilized mutant GnRH receptor (1100–1400 c.p.m. per sample) seemed to be associated specifically (in a peptide-blockable manner) with ARF and RhoA immunoprecipitates whereas specific association with these precipitates of the wild-type was negligible.

After carbachol priming, the effect of GTPγS was unaltered, that of BeF_3^- was attenuated and that of $GPPCH_2P$ was increased in a way that was 57 ± 10% inhibited by BFA. Table 1b shows that GTP analogues activated PLD in permeabilized cells and that their effects were modified by agonist priming[14–16]. After carbachol priming of 1321N1 cells, the effect of GTPγS did not change significantly but that of BeF_3^- was reduced and that of $GPPCH_2P$ was greatly increased. Agonist priming lowered the effective concentration for half maximum response (EC_{50}) for $GPPCH_2P$ from 367 ± 74 μM to 97 ± 11 μM (6% and 22%, respectively, of the potency of GTPγS (itself with an unaltered EC_{50} of 21–22 μM)). Agonist exposure causes ARF1/3 and RhoA[7,16,17] to translocate to cell membranes and the increased effect of $GPPCH_2P$ here was abrogated by BFA or C3 exoenzyme (Table 1b). Effects of AlF_4^- and BeF_3^- may involve trimeric, but not small, G proteins[13,18] (although selectivity may be modified *in vivo*). In contrast, βγ-methylene analogues of GTP may retain a substantial fraction of the potency of GTPγS at ARF, Rab5 and other small G proteins, despite being very weak activators of trimeric G proteins and Ras[19,20]. Indeed in an assay of [^{35}S]GTPγS association to native ARF, collected from ID9 immunoprecipitates (which were later renatured) of urea-treated extracts from carbachol-primed 1321N1-cell membranes, $GPPCH_2P$ retained 30% of the potency of GTPγS with mean IC_{50} values of 54 and 16 μM, respectively (R.M. and D.M., unpublished observations). These results concur with the idea that agonist-induced translocation of ARF/RhoA to membranes may promote their involvement in some form of complex with GPCRs, and may ultimately enhance receptor-mediated activation of PLD.

Each of the receptors that were sensitive to BFA, C3 exoenzyme or CMV5 Asn 19 RhoA or that could be co-immunoprecipitated with ARF 1/3 or RhoA contains the canonical AsnProXXTyr motif (where X represents any amino acid) in transmembrane domain VII (TMD VII), whereas the other receptors contain AspProXXTyr instead. The wild-type gonadotropin-releasing hormone (GnRH) receptor (which contains AspProXXTyr) and two mutants containing an AsnProXXTyr motif (containing mutation of Asp 318 → Asn or Asn 87 → Asp as well as Asp 318 → Asn (ref. 21)) were expressed in COS 7 cells. The wild-type receptor activated PLD linearly over 30–40 min, like the receptor in αT3-1 cells[22]. In contrast, the Asn 318 and Asp 87 Asn 318 mutants displayed initial rates of [^{3}H]phosphatidylbutanol formation that were more than 2.5-fold that of the wild-type receptor (Fig. 3a). Enhanced PLD coupling in the mutants was observed despite reduced [^{3}H]inositol phosphate production (Fig. 3b) and reduced membrane [^{125}I]buserelin binding (for which values of 589 ± 39, 230 ± 41 and 148 ± 36 fmol per mg protein B_{max} were obtained for cells expressing wild-type, single mutant and double mutant receptors, respectively). PLD-activation responses of the mutants desensitized rapidly, whereas PLC-activation responses did not. In contrast, postreceptor stimulation of trimeric G proteins with AlF_4^- caused non-desensitizing activation of both PLD and PLC in Asn 318 mutant cells. Neither altered receptor kinetics nor enhanced receptor internalization to allow contact with intracellular PLD[23] could explain the enhanced PLD coupling, as [^{125}I]buserelin association with the membrane and receptor internalization rates in wild-type and Asn 318-mutant cells were indistinguishable. In addition, 200 μM monodansylcadaverine and 30 μM monensin (which caused 70–80% inhibition of receptor internalization) increased rather than reduced, both PLD responses (data not shown).

PLD activation by the wild-type receptor was inhibited by U73122 (IC_{50} of 11 ± 1 μM) but not by BFA in concentrations up to 200 μM (these data are similar to data from αT3-1 cells), whereas the Asn 318-mutant response was sensitive to BFA (IC_{50} of 54 ± 8 μM) but not U73122 in concentrations up to 20 μM (Fig. 3c, d). ATP, an agonist for the native P_{2u} receptor (which contains the AspProXXTyr motif), and ionomycin/phorbol ester both caused BFA-resistant PLD activation (data not shown). The gain of BFA-sensitive PLD (but not PLC) activation upon replacement of the AspProXXTyr motif with AsnProXXTyr indicates that this structural motif may be important in gating ARF/Rho-mediated coupling to PLD. This hypothesis is supported by the greater sensitivity to BFA (IC_{50} 47 ± 11 μM) of PLD activation by the wild-type 5-HT_{2A} receptor (which contains AsnProXXTyr) than its Asp 376 mutant (ref. 24) (24 ± 13 inhibition at 200 μM BFA) expressed in COS 7 cells.

The importance of the AsnProXXTyr motif in the proposed linkage between receptor and small G proteins was directly indicated by co-immunoprecipitation of agonist-treated Asn 318 mutant but not wild-type GnRH receptors using ARF and RhoA antibodies (Fig. 3e). Furthermore, although GTPγS increased dissociation of [^{125}I]buserelin from both the Asn 318 mutant and the wild-type receptor in permeabilized cells, $GPPCH_2P$ was effective towards the mutant only and the inverse was true for BeF_3^- (Table 1c). The effect of GTPγS at the mutant, but not the wild-type, receptor was inhibited (by 62 ± 12%) by 50 μM BFA.

Our results suggest a model involving a previously unrecognized association between certain rhodopsin-family receptors and the small G proteins ARF and RhoA. One functional consequence of this appears to be an enhanced coupling of receptors to PLD activation. The interaction seems to occur when an AsnProXXTyr, but not AspProXXTyr, receptor motif is present and may be enhanced by agonist-induced translocation of ARF/RhoA to the plasma membrane. The form and site of interaction of small G proteins with receptors is unknown, but it seems likely that other proteins that act as adapters or regulators of small G protein function[25–28] may participate or mediate in the association. □

Table 1 Effects of GTP analogues on agonist recognition by M_3 and GnRH receptors and on PLD activation

Assay and treatment	GTP analogue		
	GTPγS	F^-	$GPPCH_2P$
(**a**) Carbachol affinity for [^{3}H]NMS-binding sites in 1321N1 cells (fold increase in IC_{50})			
Control	5.23 ± 0.97	4.06 ± 0.80	2.31 ± 0.34
Carbachol-primed	4.48 ± 0.34	1.82 ± 0.31*	3.46 ± 0.40†
(**b**) Activation of PLD in permeabilized 1321N1 cells (Increase over basal activation (%))			
Control	150 ± 9	115 ± 14	48 ± 8
+BFA		117 ± 9	31 ± 3‡
+C3 exoenzyme		95 ± 11	27 ± 5‡
Carbachol-primed	168 ± 10	78 ± 10*	110 ± 12†
+BFA		69 ± 11	38 ± 9‡
+C3 exoenzyme		85 ± 9	23 ± 7‡
(**c**) [^{125}I]buserelin dissociation rate in transfected COS7 cells (Reduction in $t_{1/2}$ of slow component (%))			
Wild-type GnRH receptor	43 ± 4	36 ± 8	12 ± 5
Asn 318 mutant	40 ± 5	7 ± 5*	38 ± 8†

GTPγS, F^- and $GPPCH_2P$ were present at 100 μM, 10 mM and 200 μM, respectively, except in **c** where 3 mM F^- and 100 μM $GPPCH_2P$ were used. BFA and C3 exoenzyme were used at 100 μM and 4.8 μg ml^{-1}, respectively. Values are means ± s.e.m., n = 4–10. Statistically significant differences (P < 0.05 by Wilcoxon test): *, less than corresponding unprimed control (**a**, **b**) or wild-type control (**c**); †greater than corresponding unprimed or wild-type control; ‡reversal of GTP-analogue effect.

Methods

Phospholipase assays. PLD and PLC activities were monitored as production of [^{3}H]phosphatidylbutanol and [^{3}H]inositol phosphates, respectively[29]. For assays of PLD activity in acutely permeabilized cells, prelabelled 1321N1 cells were primed with 100 μM carbachol (10 min, 37 °C) or control before intracellular buffer was added. This buffer contained 2 mM MgATP, 10 μM NAD, 6 μM digitonin, 30 mM butan-1-ol, and GTPγS, $GPPCH_2P$ or F^- (NaF in the presence of 30 μM $BeCl_2$) and BFA or C3 exoenzyme as required. Male rat anterior hemipituitaries were labelled for 2 h *in vitro* for assay of AT_{II}-evoked PLD activation.

Liposome treatment and transfection of 1321N1 cells. Cells were treated with lipofectamine containing C3 exoenzyme (2 μg per well, 5 h), CMV5-RhoA constructs (0.5 μg DNA per 4.5 cm^2 well, 7 h) or control. Rho-construct cells were assayed after 48 h.

Solubilization and immunoprecipitation of [^{3}H]NMS-labelled M_3 receptors and [^{125}I]TRP-labelled thrombin receptors. 1321N1 cells (preincubated with carbachol (100 μM) or the thrombin agonist Ser-Phe-Leu-Arg-Asn-NH_2 (30 μM) for 10 min) were homogenized in cold Hepes buffer with peptidase and phosphatase inhibitors. Membranes were solubilized in 5 mM CHAPS, 0.1% Na cholate and 1M NaCl for 30 min at 4 °C. An equal volume of 20% glycerol in CHAPS/cholate buffer without NaCl was added (with 0.6 mg ml^{-1} phosphatidylcholine for M_3 receptors). Supernatant was precleared and incubated (4 °C, 18 h) with sheep anti-ARF1/3 immunoglobulins (10–15 μl/ml; antigen $ARF1_{98-112}$; gift from M. J. O. Wakelam)[29] or with an immunoprecipitating rabbit anti-RhoA IgG (2–3 μg ml^{-1}; antigen $RhoA_{119-132}$; Santa Cruz Biotechnology)[10]. The ARF antiserum immunoprecipitated authentic immunoreactive ARF from CHAPS/cholate/NaCl membrane extracts (Fig. 2a) and from cytosol after its incubation with GTPγS and the addition of CHAPS/cholate/NaCl but not without this treatment (R.M. and M.J., unpublished observations). Blocking peptides were used at 6 μg ml^{-1} and control non-immune IgG at 3 μg ml^{-1}. Immune complexes were collected with protein-G–Sepharose. M_3 receptors were assayed in 10 mM $MgCl_2$, 200 mM NaCl, 10% glycerol, 3 mg ml^{-1} phosphatidylcholine, 20 mM Hepes pH 7.5, 10 nM [^{3}H]NMS (85 Ci $mmol^{-1}$, Du Pont) with or without 10 μM *N*-methyl atropine for 40 min at 37 °C, before precipitation with polyethylene glycol. Thrombin receptors were assayed in 10 mM $MgCl_2$, 150 mM NaCl, 0.25% BSA, 0.05% bacitracin, 0.1 mM 4-(2-aminoethyl)-benzene sulphonyl fluoride (AEBSF), 2 μg ml^{-1} aprotinin, 7% glycerol, 2 mg ml^{-1} phosphatidylcholine, Tris HCl (50 mM pH 7.4), [^{125}I]TRP (ref. 11; 120,000 c.p.m. per assay) with or without 300 nM unlabelled TRP, for 60 min at 4 °C.

Western blotting of immunoprecipitated extracts. Extracts from 1321N1 cells or male rat anterior hemipituitaries (preincubated with 10 μM AT_{II} for 10 min at 37 °C) were immunoprecipitated using polyclonal anti-ARF1/3 antibodies at 20 μl ml^{-1} (peptide at 8 μg ml^{-1}), polyclonal anti-RhoA IgG at 5 μg ml^{-1} (peptide at 20 μg ml^{-1}), rabbit anti-M_3 receptor serum[6] (antigen M_3 $receptor_{561-578}$) (or non-immune rabbit IgG) at 2.5 μg ml^{-1} and polyclonal anti-AT_1 receptor IgG at 5 μg ml^{-1} (antigen AT_1 $receptor_{13-24}$; 20 μg ml^{-1}; Santa Cruz). Proteins collected by protein G beads were separated by SDS–PAGE and blots were incubated with monoclonal anti-ARF IgG (clone 26, 1:200 dilution; Transduction Laboratories), or anti-Rho IgG (clone 26C4, 1:200 dilution; Santa Cruz). Detection was by horseradish-peroxidase-conjugated secondary antibody and enhanced chemiluminescence.

Modulation of agonist affinity at M_3 receptors by GTP analogues. 1321N1 cells were incubated with or without carbachol (20 μM) and/or BFA (100 μM) for 10 min at 37 °C. Carbachol displacement (10–3,000 μM) of membrane [^{3}H]NMS binding was measured in 10 mM $MgCl_2$, 100 mM NaCl, 20 mM Hepes pH 7.5, 1 nM [^{3}H]NMS, with or without GTPγS, $GPPCH_2P$ or F^-, for 60 min at 37 °C.

[^{125}I]buserelin-binding studies in transfected COS 7 cells. Receptor constructs in pcDNA1 (refs 21, 24) were transfected using DEAE dextran (20 μg DNA per 4×10^6 cells) and were assayed 72 h later. Membrane binding was assayed as described[30]. For cell-surface binding, cells in 12-well plates were incubated with ligand, with or without 3 μM GnRH, at 4 °C or 37 °C as appropriate[30]. Internalization was measured at 37 °C for 0–40 min after pre-equilibration with ligand for 90 min at 4 °C. Surface-bound ligand was dissociated with cold 0.2M acetic acid in 0.5M NaCl. Ligand dissociation in permeabilized cells was measured by prelabelling at 4 °C, and then successive incubations with medium containing 22 μM digitonin and GTP analogues at 37 °C. Initial dissociation occurred identically from wild-type and Asn 318 mutant GnRH receptors, reflecting a temperature-dependent reduction in GnRH binding. From 15–50 min, slower dissociation rates were reached and plots of $\ln\frac{B}{B_0}$ against time revealed single linear components of half-lives: wild-type: 22 ± 3 min, and Asn 318 mutant: 26 ± 3 min in controls.

Solubilization and immunoprecipitation of wild-type and mutant GnRH receptors labelled by [^{125}I]buserelin. After preincubating cells with or without 100 nM GnRH for 15 min, membranes were solubilized in 5 mM CHAPS and 1.5M NaCl (ref. 30). Extracts were adjusted to 0.5M NaCl and precleared before incubating (at 4 °C for 18 h) with the polyclonal antibodies anti-ARF1/3 (10 μl ml^{-1}) and anti-RhoA (1 μg ml^{-1}), described above, or with mouse monoclonal anti-Ras IgG (antigen $p21^{ras}$; Transduction Laboratories) or mouse monoclonal anti ERK1/2 IgG (antigen $ERK1_{325-345}$; Zymed Laboratories) as controls at 1 μg ml^{-1} (both of which are reported to recognize native conformations of their targets). Blocking peptides for the polyclonal reagents were used at 2 μg ml^{-1}. Immune complexes were collected using protein G–Sepharose, and [^{125}I]buserelin binding was measured in polyethylene glycol precipitates[30].

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